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GRADUATE COLLEGE

SPERM CELL CHARACTERISTICS IN RELATION TO ITS TRANSPORT DURING FERTILIZATION IN *PLUMBAGO ZEYLANICA* AND *NICOTIANA TABACUM*

A DISSERTATION

SUBMITTED TO THE GRADUATE COLLEGE

In partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

By

ZHAOJIE ZHANG

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SPERM CELL CHARACTERISTICS IN RELATION TO ITS TRANSPORT DURING FERTILIZATION IN *PLUMBAGO ZEYLANICA* AND *NICOTIANA TABACUM*

A DISSERTATION

APPROVED FOR THE DEPARTMENT OF BOTANY AND MICROBIOLOGY

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PREFACE

This dissertation is composed of four chapters written as manuscripts for scientific journal submission. The chapters have been formatted for publication in *Planta* (chapter 1, a slightly different version from the one accepted by *Planta*), *Protoplasma* (chapter 2, accepted), *Sexual Plant Reproduction* (chapter 3) and *Zygote* (chapter 4, published). Tables and figures are numbered independently in each chapter.

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Finally, I dedicate this dissertation to my wife, Qun Ren and my daughter, FeiFei, who gave me constant support, encouragement and love.

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ABSTRACT OF DISSERTATION

Sexual reproduction in flowering plants involves two sperm cells. One sperm cell fuses with the egg cell to form the zygote and then form embryo, the other fuses with the central cell to form the nutritive endosperm. Flowering plant sperm cells are small in size, simple in structure and unlike most sperm in biology, they are non-motile. In the current study, sperm cells of *Plumbago zeylanica* and *Nicotiana tabacum* were characterized using light microscopy, immunogold electron microscopy, cell microelectrophoresis, *in vitro* assays and biochemical assays in order to understand the mechanism of sperm cell transport as well as preferential fertilization in flowering plants.

Immunogold electron microscopy showed that in both *Plumbago* and *Nicotiana*, myosin is located on the pollen plasma membrane that surrounds sperm cells, but negligible myosin occurs directly on the sperm plasma membrane. Electrophoretic immunolocalizations corroborate that superficial myosin is negligible on *Plumbago* sperm cells. When sperm cells of *Plumbago* are injected into actively streaming *Nitella* internodal cells, however, they track actin bundles; movement is sensitive to ATP and Mg^{2+}, indicating the involvement of actomyosin interaction for this movement. Negatively-charged latex beads also move in a similar pattern when injected into *Nitella* internodal cells. Capillary microelectrophoresis indicates that sperm cells carry a maximum negative charge at pH 7.4. Chemically-fixed sperm cells are weakly positively charged and do not migrate. The negative charge is therefore

thought to promote non-specific myosin binding on the sperm cells that causes interactions with actin bands or coronas on the periphery of the egg and central cell that propel sperm to their sites of fusion.

In *Plumbago*, the sperm cell that normally fertilizes the egg has a higher calculated charge (8.277×10^3 esu/cm^2) compared with the sperm cell that fuses with the central cell (6.120×10^3 esu/cm^2). Microelectrophoresis of tobacco sperm cells was also conducted in this study. It was found that the two sperm cells of tobacco differ in cell size and surface charge. The smaller one (associated with vegetative nucleus, S_{vn}) has an average diameter of 6.73 μm, whereas the larger one (unassociated with vegetative nucleus, S_{ua}) is 7.52 μm. The surface charges for S_{vn} and S_{ua} are $2.94 \times 10^3 \pm 0.17 \times 10^3$ esu/cm^2 and $3.03 \times 10^3 \pm 0.17 \times 10^3$ esu/cm^2, respectively.

To study potential gene involvement in preferential fertilization, a protocol was developed for individually collecting two populations of sperm cells, S_{vn} and S_{ua}, from the pollen of *Plumbago zeylanica*. My goal is to improve the utility of sensitive techniques, such as immunoblotting, elicitation of monoclonal antibodies or the preparation of PCR-amplified cDNA libraries, by using the highly purified populations of sperm cells from each morphotype (S_{vn} and S_{ua}). The use of these techniques in sperm cell biology will enhance our understanding of the unique properties that the male gametes of angiosperms may possess and contribute to an understanding of the nature of preferential fertilization.

GENERAL INTRODUCTION

In flowering plants two sperm cells are involved in double fertilization, in which one sperm fuses with the egg cell to form the embryo, and the other fuses with the central cell to form the nutritive endosperm. Sperm cells, formed by mitotic division of the generative cell in the pollen grain or tube, are delivered through the pollen tube and released into the embryo sac. Sperm cells then undergo cytoplasmic fusion with their female target cells, transmitting the nucleus, and nuclear fusion occurs between the male and female nuclei.

Since sperm cells play a fundamental role in double fertilization, they have been a subject of intensive studies. The use of electron microscopy (since the late 1960's), combined with the techniques of computer-assisted three-dimensional reconstruction (since the mid 1980's), has significantly extended our knowledge of flowering plant sperm cells, including their formation, organization as a male germ unit, sperm heteromorphism and preferential fertilization. Molecular biology is an increasingly powerful tool for improving our understanding of the function of sperm cells during fertilization.

Sperm cell formation and male germ unit

The male germ lineage is formed by an asymmetrical division within the microspore. This asymmetric division, which is critical in controlling pollen cell fate (Twell *et al.*, 1998), results in the formation of a larger vegetative cell and a smaller generative cell. The generative cell detaches from the cell wall by "peeling" from the internal surface of

the pollen grain. As it enters the vegetative cytoplasm, the generative cell becomes surrounded by the vegetative cell, forming a unique "cell within a cell" structure (Russell *et al.*, 1996). As a consequence, an external membrane, the membrane of the vegetative cell, is found around the generative and later sperm cells (Fig. 1). This external membrane may play critical roles in the association of the two sperm cells and sperm cell transport inside the pollen tube.

The generative cell is usually closely associated with the vegetative nucleus. After generative cell division, sperm cells retain or reform their association with the vegetative nucleus. One sperm is consistently associated to the vegetative nucleus, and the second one is attached to the first, forming a tripartite structure called the male germ unit (Dumas *et al.*, 1984, Mogensen, 1992). The male germ unit, as a functional structure, forms a linkage between the nuclear and cytoplasmic DNA-containing compartments of male heredity so that the male germ cells and the vegetative nucleus appear to be transmitted together through the pollen tube. In tobacco, a recent study shows the male germ unit may help to prevent self-fusion of the two sperm cells during sperm transport inside the pollen tube (Tian and Russell, 1998).

Sperm cell isolation and characterization

Sperm cells of flowering plants are structurally simple, and share many common structural properties with generative cells. The newly-formed generative cell is surrounded by a wall, which disappears gradually as the generative cell is released from the intine (Russell *et al.*, 1996). Ultrastructural studies confirm that the sperm cells, lacking cell walls, are complete cells, rather than naked nuclei. These cells have plasma

membranes, nuclei and their own complement of cytoplasmic organelles (Jensen and Fisher, 1968, 1970; Zhu *et al.* 1980; Russell and Cass, 1981). The cytoplasm of the male gametes generally contains of mitochondria, endoplastic reticulum, ribosomes, and vesicles (Yu *et al.* 1989). Sperm cells were traditionally regarded as transcriptionally quiescent, because the sperm nuclei often possess densely stained heterochromatic granular chromatin (Knox *et al.*, 1988). However, Zhang *et al.* (1993) and Matthys-Rochon *et al.* (1994) showed that sperm nuclei are transcriptionally active, display electrophysiological activity and appear to be fully physiologically competent. A male gametic cell-specific gene was identified recently and shown to express in the male gametic cells of *Lilium longiflorum* (Xu *et al.* 1999).

Isolation of sperm cells has been reported in a number of species of flowering plants, since first reported by Cass (1973; for review: Southworth and Knox, 1988; Russell, 1991). Sperm cells have been reported to be obtained from either tricellular or bicellular pollen. For tricellular pollen, sperm cell can be obtained from pollen grain or pollen tube. For bicellular pollen, however, sperm cells can only be obtained from pollen tubes, which has been successful using either *in vitro* (Southworth and Knox, 1988) or *semi-vivo* culture (Shivanna *et al.*, 1988, Tian and Russell, 1997). Two general techniques have been conducted for sperm cell release from pollen grains or pollen tubes: Osmotic shock or mechanical rupture, both of which use stress to break the pollen plasma membrane.

Separation of sperm cells from pollen cell walls can be achieved by filtration with a 5 to 50 μm nylon filter. Released sperm cells have frequently been purified using centrifugation on a discontinuous gradient or filtration using a polycarbonate filter.

Viability of sperm cells has been evaluated using exclusion of polar dyes e.g., Evans blue, fluorochromatic reaction (FCR) and other techniques. Evans blue penetrates and stains only those cells that are dead or damaged; viable cells prevent the dye from entering and so remain unstained (Gahan 1984). fluorescein diacetate (FDA) is easily absorbed by living cells and becomes fluorescent only if esters are cleaved from the fluorescein molecule and the cell is intact enough to retain the polar dye (Heslop-Harrison *et al.*, 1984).

For detailed biochemical and molecular analysis of sperm cells, such as gene involvement in preferential fertilization and differences in gene expression of different sperm cells (S_{vn} and S_{ua}), highly purified two populations of sperm cells (S_{vn} and S_{ua}) are needed (Chapter 4).

Sperm cell heteromorphism and preferential fertilization

Differences between the two sperm cells within a pollen grain or tube have been reported in a number of species of angiosperms (Russell, 1991). In well-described cases, these differences mainly include: (1) differences in cytoplasm (cytoplasmic heterospermy); and (2) differences in nuclear contents ("nuclear heterospermy"). The most common form of cytoplasmic heterospermy consists of iniquities in the number of mitochondria between the two sperm cells, with the S_{vn} usually containing more mitochondria (Russell, 1992). Nuclear heterospermy has been reported only in *Zea mays*, where B chromosomes often do not separate during generative cell division, resulting in aneuploid sperm cells (Roman, 1948). In *Plumbago zeylanica*, both mitochondria and plastids differ between the two sperm cells. The S_{vn} contains no

plastids and more than 200 mitochondria, whereas the S_{ua} contains an average of 24 plastids and less than 50 mitochondria. Sperm heteromorphism may relate to preferential fertilization, in which one sperm cell has a greater likelihood of fusing with the egg (Russell, 1992). In *Plumbago zeylanica*, for example, the fusion between the egg and the plastid-rich sperm cell (S_{ua}) occurs in 94% of the cases examined (Russell, 1985). Cell microelectrophoresis of sperm cells of *Plumbago zeylanica* showed that the two sperm cells have different surface charge densities (chapter 1), indicating that the surface charge of the cells may also be involved in gamete recognition and preferential fertilization.

Sperm cell mobility and transport

Unlike non-seed plants and some of the gymnosperms, sperm cells in flowering plants are non-motile, passive participants in their transport to the female reproductive cells. Morphological differences between the motile and non-motile sperm cells are significant, but recent study (Southworth and Cresti 1997) revealed that their cytoskeleton pattern and nuclear structure are similar. The motile sperm cells of lower vascular plants are either biflagellate or highly multiflagellate (Table 1). In *Lycopodium* and *Selaginella* the sperm are biflagellate and in this respect are quite unlike the prevailingly multiflagellate sperm developed in all other "lower vascular plants", such as Pterophytes and in *Ginkgo* and the cycads (Gifford and Foster, 1989). The sperm of many algae are also biflagellated, which are similar as the sperm of *Lycopodium* or *Selaginella*.

Biflagellate sperm

In *Lycopodium*, the sperm are blunt-ended, fusiform cells, about 10 μm long and 5 μm wide. The flagellae trail behind the cell as it swims. A mitochondrion inside the cell is closely associated with a multilayered structure with microtubules forming a supportive spline. A large amyloplast, containing starch grains, occurs at the posterior end. The basal body of each flagellum lies close to the multilayered structure (Robbins and Carothers 1978).

In *Selaginella* a mature swimming sperm is about 25 μm long and about 0.50 μm wide. One of the two flagella is attached at the anterior end and the other attached at the middle of the sperm. The mitochondrion is relatively large and occupies the anterior half. A partial sheath of microtubules extends the entire length of the sperm, functioning possibly as a support mechanism (Robert, 1974).

Multiflagellate sperm

The structure of multiflagellate sperm differs from species to species. In *Equisetum* each sperm has about 120 flagellae. The flagellae beat with a continuous, traveling, helical wave, propelling a sperm forward in a unidirectional, helical path (Bilderback *et al.*, 1973). The sperm of *Pteridium* resembles a short corkscrew, with the flagellae attached to a lamellar band in the anterior gyres of the helix. The flagellae beat in a coordinated wavelike fashion, thrusting the sperm forward and also causing it to rotate (Duckett and Racey 1974).

The sperm of cycads resemble those of *Ginkgo*, aside from their larger size. For example, the sperm of *Zamia* attains a diameter of 180 μm, while the sperm of *Ginkgo* is only about 80 μm in diameter (Norstog, 1967). It is observed that the two sperm

move freely for some time within the intact pollen tube. In *Ginkgo* usually only one of the two sperm produced by a pollen tube enters an archegonium; the other one, if it enters the same archegonium, soon degenerates and is eventually absorbed (Lee, 1955). *Ginkgo* and cycads also represent the only known plants that form both flagellated sperm and pollen tubes (Friedman 1993).

Non-motile sperm

In contrast with the large sperm found in *Ginkgo* and the cycads, the sperm in other extant gymnosperms as well as in angiosperms are small and devoid of flagella. These non-motile sperm cells are transported through the pollen tube then released into the embryo sac (Russell, 1996). One question remaining to be resolved is how the non-motile sperm cells migrate inside pollen tubes and later within the intercellular space between the egg cell, synergid and central cell to the fusion sites and fuse exactly with their target cells. Several hypotheses have been proposed, including active migration, fountain flow, passive migration and actomyosin interaction.

Active migration. The vermiform appearance of the sperm cells in several plants led Nawaschin (1898, cited from Kapil and Bhatnagar 1975) to consider them to be weakly motile. An amoeboid independent movement of generative cell and sperm cell was described in pollen tubes of *Galanthus nivalis* (Steffen 1953). Wulff (1933) observed that the cytoplasm in pollen tubes runs in various directions but the sperm cell always maintained a consistent forward motion. It appeared unlikely that cytoplasmic streaming was responsible for their movement. Furthermore, the presence of microtubules in sperm cells of *Beta* (Hoefert, 1969) was oriented parallel to the long axis of the cell -- a condition which is considered responsible for locomotion in

unicellular animals, and which has been correlated with possible motility of the sperm cell. In contrast, such microtubules described in the generative cell of *Haemanthus* (Sanger and Jackson, 1971) and sperm cells of barley (Cass, 1973) are believed to mediate changes in cell shape rather than bringing about locomotion.

Fountain flow. Fisher and Jensen (1969) reported that the ejection of the contents of the pollen tube into the embryo sac of cotton, like a fountain flow, pushes the cytoplasm of synergid aside, rather than ahead of the emerging pollen tube cytoplasm. The pollen tube contents entered the synergid earlier follows this lateral movement and deposits aside of the pollen tube tip, but that ejected later moves further into the synergid. As a result, the pollen tube contents farthest from the pollen tube tip, which includes the two sperm cells and vegetative nucleus, may actually penetrate further into the embryo sac.

Passive migration. Strasburger (1884, cited from Kapil and Bhatnagar 1975) believed that the transport of sperm was accomplished passively by cytoplasmic streaming. Experimental studies of living sperm cells of barley (Cass, 1973) also confirmed the sperm cells are non-motile and their transport is passive.

Actomyosin interaction. Recent evidence has suggested that actomyosin interactions are involved in sperm cell transport both inside the pollen tube and inside the embryo sac. Actin bundles found in the cortical cytoplasm of the pollen tube are axially aligned in the pollen tube cytoplasm (Tang *et al.* 1989a). Using anti-myosin antibodies, myosin is localized on generative cells, the predecessor of sperm cells and the vegetative nucleus (Heslop-Harrison and Heslop-Harrison 1989b, Tang *et al* 1989b, Miller *et al.* 1995, Tirlapur *et al.* 1995, 1996). Such actomyosin patterns suggest that

interactions between myosin and actin filaments may provide sufficient force to move sperm cells inside the pollen tube. The role of actomyosin interactions between the male germ unit and cortical cytoplasmic actin is further supported by cytochalasin B inhibition, which is believed to depolymerize actin filaments (Lancelle and Hepler 1988, Heslop-Harrison and Heslop-Harrison 1989a). Inside the pollen tube, the male germ cells are surrounded by the inner pollen plasma membrane that separates these cells from the interior of the pollen cytoplasm (Russell 1994). It was unclear, however, whether myosin was present on the sperm cells or associated with the surrounding pollen tube plasma membrane. This may have important ramifications in their later function (Russell, 1994). Myosin associated with the surrounding tube plasma membrane would be expected to function optimally within the intact pollen tube; however, this membrane is typically lost upon arrival in the embryo sac when the pollen tube discharges its contents (Huang and Russell, 1992) and during sperm cell isolation (Russell 1991). Observations indicate that in addition to this loss of membranes, the actin bundles characteristic of the pollen tube are concomitantly disassembled, resulting in a bilateral breakdown of the prior transport mechanism.

Upon pollen tube discharge, the cytoplasmic organization of the synergid is dramatically altered. In *Nicotiana*, for example, two actin bands or "coronas" are formed; one occurs between the egg and central cell, terminating near the central cell nucleus, and the other occurs at the chalazal end of the receptive synergid, terminating near the egg nucleus (Huang and Russell, 1994). These coronas appear to trace the pathway taken by the sperm cells during their passage to the egg and central cell. Similar bands were also found in maize (Huang and Sheridan, 1994). In *Plumbago*,

which lacks synergids, only one such band was observed, but this also terminates near the egg and central cell nuclei (Huang and Russell, 1993). The potential for continued actomyosin migration of the sperm cells to the female target is still possible, if myosin is present directly on the sperm cells. The assessment of the exact location of myosin on the sperm cells has thus become an important issue for understanding the mechanism of sperm cell transport (Russell, 1996).

Plant myosins and sperm cell transport

In contrast to the conservative nature of actin, myosins have been found to be a superfamily of proteins characterized by the presence of a conserved ~80 kDa motor domain attached to a variety of structurally distinct tail domains. This superfamily consists of at least 13 classes of myosins (Cope *et al.*, 1996). Knowledge of plant myosins, however, is still very limited. Sequence analysis of plant myosin genes places the known products in only two of those classes. Class V is represented in yeast and animals as well as plants and Class VIII, is a group which so far is plant-specific (Knight and Kendrick-Jones 1993; Kinkema and Schiefelbein 1994, Kinkema *et al.* 1994; Plazinski *et al.* 1997).

Using antibodies to myosin II, a 175 kDa protein in *Nicotiana alata* has been identified that localized as large punctuate spheres thought to represent organelles (Tang *et al.*, 1989b). It was not until 1992 that myosin was first partially purified from the pollen tubes of lily (Kohno *et al.*, 1992). SDS-PAGE shows that fractions have molecular masses of 100, 120, and 140 kDa, but only the component of 120 kDa showed an ATPase activity. A myosin with a molecular weight of 170 kDa was further

purified from lily pollen tubes (Yokota and Shimmen, 1994). *In vitro* motility assays suggested that this myosin produced the motive force for cytoplasmic streaming in pollen tube of lily.

Three classes of myosins, myosin I, II and V, were later identified in pollen tubes of *Lilium longiflorum* and *Nicotiana alata* (Miller *et al.*, 1995). .Myosin I was localized to the plasma membrane, larger organelles, the surface of the generative cell and the vegetative nucleus, whereas, myosin V labeled vegetative cytoplasm in a punctuate fashion, resembling smaller organelles. Myosin II was localized in a punctuate fashion on the larger organelles. These results suggest that myosin I may move the generative cell and vegetative nucleus unidirectionally through the pollen tube to the tip, while myosin V moves the smaller organelles. Myosins I and II appear to move the larger organelles bidirectionally.

Objectives of the present study

The main objective of this dissertation is to study the sperm cell surface characteristics in relationship to sperm transport during fertilization. According to the current model, actomyosin interactions may play a key role for the sperm cell transport (Huang and Russell, 1994; Russell, 1996). The exact location of myosin on the sperm cells, however, remains to be resolved. In this study, different techniques such as immunoelectron microscopy, *in vitro* assays, and cell microelectrophoresis, were used to assess the presence and location of myosin on isolated sperm cells in relation to this model.

Another objective is to study preferential fertilization on molecular level. As a first step, highly purified viable sperm cells of both the S_{vn} and S_{ua} were collected as separate populations in order to study potential gene involvement in preferential fertilization. The two populations of viable sperm cells (S_{ua} and S_{vn}) collected from pollen of *Plumbago zeylanica* represent the two sperm cells that participate in the separate fusion events of double fertilization, in which the preferential fertilization has been best described.

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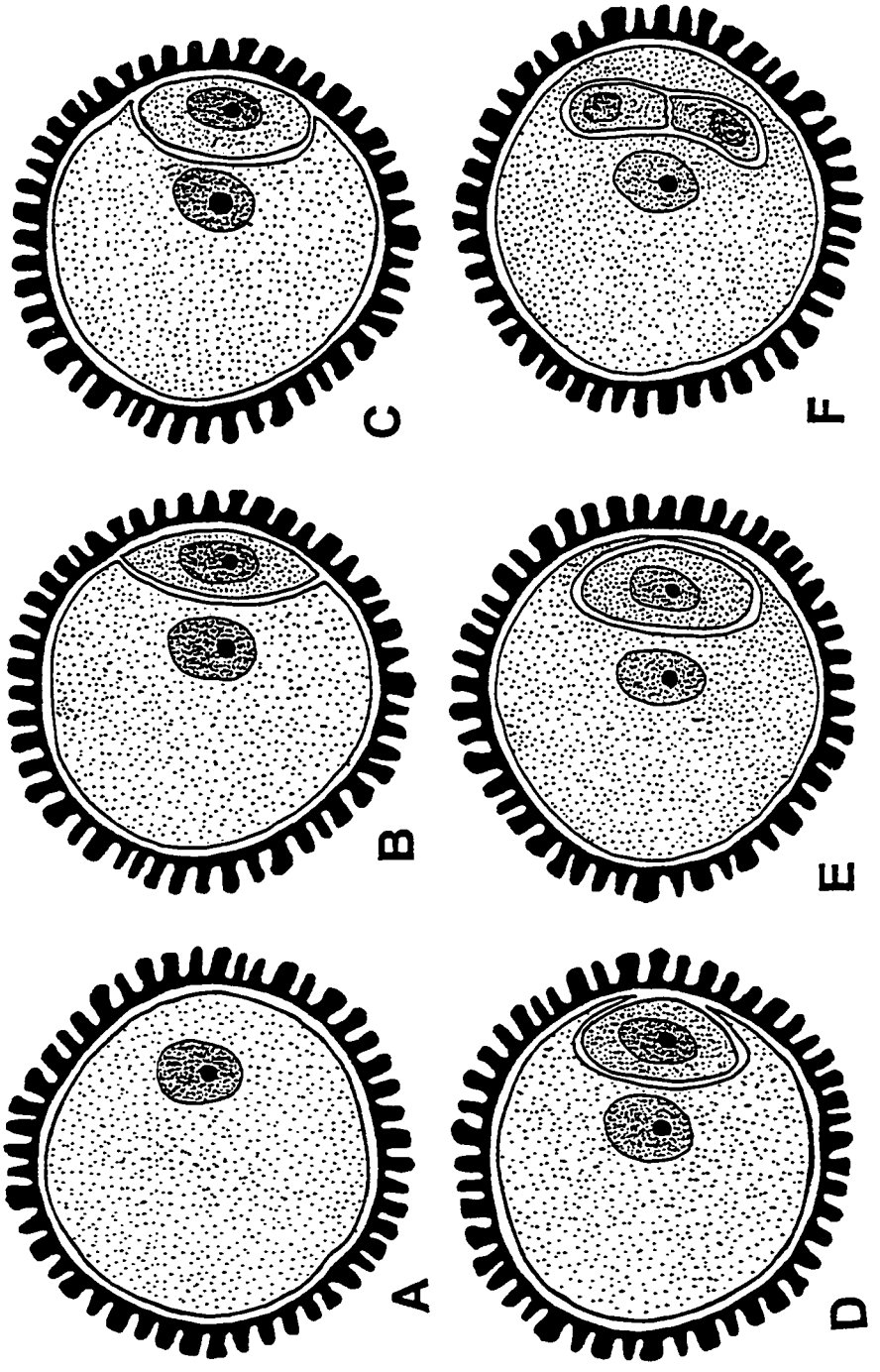
Figure 1. Development of angiosperm pollen, showing the generative cell and later the sperm cells are surrounded by an inner pollen membrane.

A. B. An asymmetric mitotic division produces a larger vegetative cell and smaller generative cell. **C-E.** The generative cell detaches from the cell wall by "peeling" from the internal surface of the pollen grain. As entering the vegetative cytoplasm, the generative cell becomes surrounded by the vegetative cell, forming a unique "cell within a cell" structure, as a consequence, an external membrane, the membrane of the vegetative cell, is formed around the generative cell. **F.** A mitotic division of the generative cell forms the two sperm cells, which are still surrounded by the external membrane.


C
F
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E
A
D

Table 1. Sperm cell types according to mobility

Sperm		Plants	
Motile	Biflagellate	Algae	Phaeophyta
			Chlorophyta
		Bryophytes	Hepatophyta
			Anthocerophyta
			Bryophyta
		Pteridophytes	Lycophyta
	Multiflagellate	Pteridophytes	Psilophyta
			Sphenophyta
			Pterophyta
		Gymnosperms	Ginkgophyta
			Cycadophyta
Non-motile		Gymnosperms	Coniferophyta
			Gnetophyta
		Angiosperms	Magnoliophyta

CHAPTER 1

Sperm cell surface characteristics of *Plumbago zeylanica* in relation to transport in the embryo sac

Abstract. Generative and sperm cells are associated with myosin during pollen tube elongation, promoting interactions with actin that maintain the position of the non-motile sperm cells with relation to the tip of the tube. This myosin appears to be associated with an external membrane of pollen origin that is lost after tube discharge. Lack of motility on actin matrices, absence of myosin label in immunoelectron microscopy and inconclusive electrophoretic immunolocalizations suggest that superficial myosin is negligible directly on *Plumbago* sperm cells. When sperm cells are injected into actively streaming *Nitella* internodal cells (a model actomyosin system), however, sperm cells track actin bundles; movement is sensitive to ATP and Mg^{2+}. Negatively charged latex beads also move. Capillary microelectrophoresis indicates that sperm cells carry a maximum negative charge at pH 7.4. Chemically-fixed sperm cells are weakly positively charged and do not migrate. The negative charge is therefore thought to promote non-specific myosin binding on the sperm cells that causes interactions with actin bands or coronas on the periphery of the egg and central cell that propel sperm to their sites of fusion. In *Plumbago*, the sperm cell that normally fertilizes the egg has a higher calculated charge (8.277×10^3 esu/cm^2) compared with the sperm cell that fuses with the central cell (6.120×10^3 esu/cm^2).

Key words: Fertilization - Male germ unit - Myosin - *Plumbago* - Sperm transport

Introduction

Sperm cells of flowering plants appear to be transported through the pollen tube principally by means of actomyosin interactions. Throughout their transmission in the pollen tube, sperm cells of flowering plants are not independently motile and are closely associated with axial bundles of F-actin in the cortex of the pollen tube cytoplasm. Prior research has indicated that the surface of the generative cell--the predecessor of the sperm cells--is labeled with anti-myosin antibodies (Heslop-Harrison and Heslop-Harrison 1989; Tang et al. 1989; Miller et al. 1995; Tirlapur et al. 1995, 1996), as are the pollen wall generating vesicles, known as P-particles (Heslop-Harrison et al. 1997). This suggests that generative cell and P-particle associated myosin may interact with actin filaments, at least in part, to position these bodies within the elongating pollen tube and thus provide for their translocation. There is also emerging evidence that microtubules may contribute to a slower pattern of forward movement (Heslop-Harrison and Heslop-Harrison 1996).

The exact location of the myosin layer associated with the generative cell during its transmission in the pollen tube is unclear when examined with epifluorescent microscopy using an anti-myosin antibody (Heslop-Harrison and Heslop-Harrison 1989; Tang et al. 1989). Labeling with immunogold probes provides greater resolution on sectioned generative cells but sparser label (Tirlapur et al. 1996) and this method also seems inconclusive with respect to the membrane surfaces involved. Whether myosin is present on the sperm cells or associated with the surrounding pollen tube plasma membrane may have important ramifications in their later function

(Russell 1994). Myosin associated with the surrounding tube plasma membrane would be expected to function optimally within the intact tube, however, this membrane is typically lost upon arrival in the embryo sac when the pollen tube discharges its contents (Huang and Russell 1992). Rapidly frozen embryo sacs depicting this latter stage provide evidence for the loss of this membrane (Huang et al. 1993a). Observations indicate that in addition to this loss of membranes, the actin bundles characteristic of the pollen tube are concomitantly disassembled, resulting in a bilateral breakdown of the prior transport mechanism.

Upon pollen tube discharge, the cytoplasmic organization of the tube is dramatically altered. Actin in the embryo sac near the egg cell forms actin bands or "coronas" at this stage, which are temporally associated with the arrival of the sperm cells. Two such coronas occur in normal embryo sacs: one extends from the mid-lateral region of the synergid to near the egg nucleus, whereas the other extends from the mid-lateral region outside the egg cell, to near the polar nuclei (Huang and Russell 1994). These coronas follow the expected pathways of sperm cell transport upon sperm cell delivery in the embryo sac. Only one such band is found in the embryo sac of *Plumbago*, which lacks synergids, but this also terminates near the egg and central cell nuclei (Huang et al. 1993b). Since actomyosin interactions with these actin bands are suspected in short distance transport of cells after pollen tube discharge, the loss of the pollen plasma membrane surrounding the sperm cells raises critical questions about whether myosin is directly present on sperm cells or whether some other mechanism may be needed to move the deposited male gametes within the

embryo sac to their sites of fusion. The unique environment of the embryo sac must also be considered, as it may protect specific classes of myosin or preferentially retain sperm-associated myosin, promoting migration.

Synergid-based actin bands associated with double fertilization are also reported in maize (Huang and Sheridan 1994) and appear in other flowering plants, according to prior published electron micrographs (Russell 1994). If myosin is present directly on the sperm cells, the potential for continued actomyosin migration of male gametes to the female target nuclei is possible; if not, reassessment and refinement of such a model will be required (Russell 1996). The current study assesses the presence and location of myosin on isolated sperm cells in relation to this model using immunoelectron microscopy, *in vitro* assays, cell surface charge estimations, dot blots and immunoelectrophoresis.

Materials and methods

Preparation of sperm cells. Plants of *Plumbago zeylanica* L. were grown in a greenhouse in the Department of Botany and Microbiology, University of Oklahoma. Pollen was collected from flowers into a Petri dish and then used immediately or kept at -20 °C until use. Sperm cells were isolated according to the protocol of Russell (1986). Briefly, the pollen was immersed into 20% (w/v) sucrose dissolved in water. The pollen grains were allowed to burst for 20 min. Then the solution was rinsed through a 53 μm nylon filter to remove the pollen walls, layered over a 30% (w/v) sucrose solution in a centrifuge tube, and centrifuged at 1,500 × g for 8 min. The pellet was rinsed again with 20% sucrose, layered over 30% sucrose and centrifuged

at 1,500 × g for 7 min to eliminate the cytoplasmic organelles of pollen vegetative cells. For microelectrophoresis, the sperm cells were resuspended in a low ionic buffer (LIB) containing 10 mM NaH_2PO_4, 0.3 mM citric acid and 14% sucrose, pH 7.4. Microelectrophoresis of sperm cells at different pH was also conducted, by adjusting the pH of the buffer with dropwise addition of 1N HCl or 1N NaOH.

Immunogold electron microscopy. Isolated sperm cells were blocked 15 min with 1% bovine serum albumin (BSA) and 2% normal goat serum in phosphate buffered saline (PBS) (pH 7.3) containing 15% sucrose and incubated 1.5 h in an anti-myosin antibody (M-7648, Sigma Chemical Co., St. Louis, MO) diluted 1:10 with blocking solution. After 3 washes with blocking solution, the sample was incubated 1 h in a 1:5 dilution of 10 nm gold-conjugated goat anti-rabbit IgG (EY Labs, Inc., San Mateo, CA) in blocking solution. The specimen was then rinsed in PBS, fixed with 2% glutaraldehyde for 40 min, dehydrated using a graded ethanol dehydration series and embedded with Embed 812 resin (Electron Microscopy Sciences, Fort Washington, PA). Thin sections were stained using uranyl acetate for 25 min and lead citrate for 1 min. Controls were prepared by omitting primary antibody.

Injection of sperm cells and latex beads into Nitella internodal cells. The charophytic alga *Nitella* was collected from Chickasaw National Recreation area (Sulphur, Oklahoma), cultured in glass containers containing soil, water from the site and distilled water, and was aerated using an aquarium pump. The containers were cultured under fluorescent lamps (25 °C) with a 14 h/day lighting regime.

Selected long internodal cells of *Nitella* were cut at both ends and incubated in mobility buffer (25 mM KCl, 4 mM $MgCl_2$, 4 mM ethylene glycol-bis(ß-aminoethyl ether) n,n,n',n',-tetraacetic acid (EGTA), 5 mM imidazole, 200 mM sucrose and 1 mM ATP, pH 7) (Kohno and Shimmen 1988, Kachar et al. 1993). Sperm cells and/or latex beads (6 μm Polybead polystyrene spheres, Polysciences, Inc Warrington, PA) were injected into the internodal cell through one end of the cell using a pipette with a tip diameter of approximately 500 μm. Both ends of the internodal cell were then ligated with strips of polyester threads (Shimmen and Yano 1984). Some experiments were conducted after flushing the cytoplasm from the cell; others used cells with much of the cytoplasm remaining. *Ex vitro* experiments were also conducted using removed actin bundles. Movement of sperm cells and latex beads was observed using an inverted or compound phase contrast microscope and recorded using a video camera attached to a video cassette recorder.

Cell microelectrophoresis. Microelectrophoresis of sperm cells was based on the methods of van Oss and Fike (1979). Suspended sperm cells in buffer (Russell 1986) were placed into the lumen of a capillary (0.6 mm internal diameter × 9.2 cm long), which was coated on the inside with several layers of a 2% agarose solution; the ends of the capillary were sealed with a gel-plug made from the same agarose solution. The capillary was then suspended between two beakers, immersed in the buffer with an electrode in the beaker (Fig. 1). Selected voltages were applied between the electrodes with constant current, and the mobility of sperm cells was observed using an inverted phase contrast microscope. Images were recorded on videotape using a

high sensitivity television camera and a video recorder. The velocity was measured using the recorded images, and electrophoretic mobility was calculated. To reliably identify the two sperm cells, single pollen grains were burst in a droplet of 20% sucrose, and the two sperm cells were transferred by microinjector into buffer in the center of the optical field within a special chamber constructed using 1.5 × 60 mm sheets of 17 μm thick glass glued with cyanoacrylate. This chamber allowed the relative electrophoretic mobility of paired sperm cells to be compared, while providing the optical properties required to identify the sperm cell types (Russell 1991) using a high magnification lens.

Immunoelectrophoresis. Isolated sperm cells were incubated in a rabbit IgG antibody elicited to skeletal and smooth myosin (M-7648, Sigma) at a dilution of 1:10 with phosphate buffer for 1 h at room temperature. After rinsing with buffer, some samples were also incubated in FITC-conjugated goat anti-rabbit IgG secondary antibody (F-0382, Sigma), diluted 1:100 with buffer for 1.5 h and then rinsed with buffer. Electrophoretic mobility was determined for: (1) samples without antibody, (2) samples with primary antibody, and (3) samples with both the primary and secondary antibodies.

Fixation of sperm cells and latex beads. Isolated sperm cells or latex beads were fixed with 2.5% glutaraldehyde (in 10 mM phosphate buffer containing 8% sucrose, pH 7) for 2 h, then washed 2 times with LIB (pH 7.4). The sperm cells or latex beads were then resuspended in LIB and used for microelectrophoresis.

Electrophoretic mobility and surface charge calculations. The electrophoretic mobility of individual cells was calculated by the equation:

$$\mu = (d/t) / (V/D)$$

where μ is the electrophoretic mobility, d is the distance (in μm) traveled by cells during measurement, t is time (in sec) required by cells to travel distance d, V is voltage applied to the electrodes, and D is the length of the capillary (in cm).

Surface charge density was calculated using the general formula of the Smouchowski equation (Levine et al. 1983):

$$\sigma = \mu\eta\kappa$$

where σ is the surface charge density in electrostatic units (esu) of charge per unit area (cm^2), η is the viscosity coefficient, and κ is the reciprocal of the Debye-Hückel distance, defined as:

$$\kappa = 10^8 \cdot c^{1/2} / 3.04\ cm^{-1}$$

where c is the electrolyte concentration in moles. The calculated value of κ for this experiment was 3.5×10^6 cm^{-1}, and the value of η was 1.746×10^{-3} poise, as measured using a Cannon-Fenske viscometer.

Dot Blotting. The cytoplasm of *Nitella* internodal cells and *Plumbago* pollen was used to test for the presence of soluble myosin. For *Nitella*, internodal cells were cut at both ends and immersed into Tris-buffered saline (TBS) containing 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin and 20% (w/v) sucrose. A pair of forceps was used to gently extract the cytoplasm. Pollen cytoplasm of *Plumbago* was obtained using the same buffer as *Nitella* internodal cells.

Both extracts were collected into separate centrifuge tubes and centrifuged 30 min at 2,000 × g. The pellets were discarded, and the supernatants were collected and spun at 150,000 × g for 1 h to remove any remaining cell membranes. A 1 µl drop of the final supernatants was then spotted directly on a nitrocellulose membrane. The membrane was immersed in TBS containing 0.05% Tween 20 (TBST) with 5% dry milk for 30 min, incubated in anti-myosin antibody (M-7648, Sigma) diluted 1:200 in TBST for 1 h, washed 3 times with TBST, and incubated with goat anti-rabbit IgG secondary antibody conjugated with alkaline phosphatase (Bio-Rad Laboratories, Hercules, CA) diluted 1:3000 in TBST for 40 min. After 3 washes with TBS, an alkaline phosphatase conjugate substrate kit (Bio-Rad Laboratories) was used to perform a color reaction. Controls were simultaneously run with omission of the primary antibody or replacement of the extract solution with buffer.

Results

Attempts to demonstrate myosin on the surface of isolated sperm cells. Isolated *Plumbago* sperm cells did not migrate on reconstituted actin bundles using an *in vitro* movement sustaining buffer augmented with Mg^{2+} and ATP, although other organelles (both of *Nitella* and pollen tubes) displayed slow movement. This was also true when isolated sperm cells and organelles were placed with freshly isolated actin bundles from vigorously streaming internodal cells of the alga *Nitella*, which is a model system for actomyosin-based cyclosis (Kachar and Reese 1988). When injected into *Nitella* long internodal cells from which the cytoplasm had been flushed, the sperm cells remained stationary. In all conditions, the organelles of *Nitella* sustained at least

weak movement; the sperm cells however did not migrate, even when organelles were moving rapidly along actin bundles. Immunogold labeling using anti-myosin antibodies resulted in few gold particles associated with the surface of the sperm cells, although some organelles of the pollen grain co-isolated in the pellet were heavily labeled (Fig. 2). When gold label was present, it seemed to be localized only on membranes adherent to the sperm cells and not to the sperm plasma membrane itself. Microelectrophoretic data, which uses the altered mobility of cells bound to immunoglobulin as a sensitive probe for immunological binding (Cohly and Das 1994), provided inconclusive evidence for anti-myosin antibody labeling under appropriate conditions (Fig. 3).

Sperm cells injected into the streaming cytoplasm of Nitella. Isolated sperm cells injected into actively streaming cytoplasm of *Nitella* successfully track actin bundles and migrate in contact with bundle fibers, as indicated in Fig. 4. When sperm cells are no longer in contact with the cortical cytoplasm, they travel at approximately one-quarter of the prior rate. As a control, 6 μm latex beads were injected into the cytoplasm of *Nitella*. Figure 5 indicates that these latex beads also displayed movement in the cortical cytoplasm while attached to actin bundles and traveled more slowly (approximately one-third of the previous velocity) when not attached. The slower velocity observed when sperm cells and latex beads are not tracking actin filaments is presumably generated by their movement in association with large actomyosin-based organelles, including endoplasmic reticulum, that may create cytoplasmic drag (Kachar and Reese 1988). Since the movement of latex beads, in

particular, appeared to reflect the characteristics of active actomyosin translocation in the absence of native myosin, the possibility of myosin being acquired from the cytoplasm of *Nitella* was examined.

To prevent the non-specific binding of myosin, the latex beads were preincubated in blocking solutions. These solutions included BSA, casein hydrolysate and other non-specific proteins typically used in immunocytochemistry. The treated latex beads were injected into the cytoplasm of vigorously streaming internodal cells. The treated beads moved slowly and did not track along actin filaments, appearing instead to move slowly in the current of the streaming cytoplasm. After 15 min, the latex beads gradually began to track actin bundles. Presumably, this occurred as the weakly-bound blocking proteins were lost from the surface of the beads. Incubation of latex beads in positively-charged protein (poly-L-lysine) completely inhibited movement. These observations are consistent with the presence of actomyosin tracking if the myosin is acquired from the *Nitella* cell cytoplasm. The acquired myosin, upon binding, apparently promotes movement. The presence of electrically-charged regions on the surface of these cells and the beads was suspected to be the cause of nonspecific binding and was examined using microelectrophoresis.

Surface charge of sperm cells. Sperm cells, pollen organelles, and latex beads migrate toward the positive pole of the capillary microelectrophoretic apparatus, indicating that they all carry a net negative charge on their surfaces. Sperm cells had an average electrophoretic mobility of 1.27 $\pm$ 0.23 (μm/sec)/(V/cm). The velocity was directly variable with voltage, supporting the charge basis of this movement. The

calculated surface charge density of sperm cells was $7.403 \times 10^3 \pm 1.341 \times 10^3$ esu/cm^2.

This negative charge in isolated sperm cells appears to be maintained only in living cells, since fixation of the sperm cells caused them to migrate toward the negative pole of the electrophoretic apparatus. Similar treatment of latex beads does not cause any change of their electrophoretic mobility. The apparent optimum for electrophoretic mobility of sperm cells is approximately pH 7.4, as shown in Fig. 6.

Electrophoretic mobility and surface charge density of sperm cell types. Populations of sperm cells display a bimodal distribution of electrophoretic mobilities (Fig. 7). This behavior is also evident when comparing the mobility of individual cells (Fig. 8). The electrophoretic mobility of the more rapidly moving population of sperm cells is approximately 1.42 (μm/sec)/(V/cm), whereas the more slowly moving population has a mode of electrophoretic mobility of approximately 1.05 (μm/sec)/(V/cm). The average mobilities of the two sperm cell populations differ by as much as 35%. Based on these mobilities, the more slowly moving cells have a calculated surface charge density of 6.120×10^3 esu/cm^2, and the more rapidly moving sperm cells have a charge density of 8.277×10^3 esu/cm^2.

Electrophoresis of paired sperm cells from single pollen grains further reveals that the two types of sperm cells, the S_{un} (sperm cell unassociated with the vegetative nucleus) and the S_{vn} (sperm cell physically associated with the vegetative nucleus) display different electrophoretic mobilities. The S_{un}, which is smaller and eventually

fuses with the egg cell, consistently moves faster than the larger S_{vn}, which fuses with the central cell.

Soluble myosin identification. Dot blots display color reactions using extracts from both *Nitella* internodal cytoplasm and *Plumbago* pollen cytoplasm (Fig. 9), indicating that free myosin is present in the pollen of *Plumbago*, as well as *Nitella* internodal cytoplasm. No observable staining was seen in controls.

Discussion

The movement of angiosperm generative cells, and presumably sperm cells, appears dependent, at least in part, on actomyosin interactions during their descent in the pollen tube (Pierson and Cresti 1992). Myosin associated with the male reproductive cells appears to reside on the cytoplasmic surface of the pollen tube membrane surrounding these cells. This plasma membrane, which originates with the formation and immersion of the generative cell (Russell et al. 1996), represents an inner pollen plasma membrane that is separate from the external plasma membrane of the pollen (Russell 1994). The earliest indication in the published literature that myosin is located on the ensheathing pollen plasma membrane is reported by Heslop-Harrison and Heslop-Harrison (1989), who noted that only some of the generative cells they examined were labeled with anti-myosin antibodies; these labeled cells appeared to be associated with an external membrane. When generative cells were isolated, however, some of the cells appeared to lack this membrane, and when absent, the cells were essentially unlabeled (Heslop-Harrison and Heslop-Harrison 1989). The loss of this external cell membrane occurs just prior to normal fertilization,

presumably during pollen σtube discharge (Russell 1994) and during sperm cell isolation (Russell 1991). Isolated sperm cells seem to lack functional myosin according to the current results, and immunoelectron microscopic observations indicate that only a small amount of myosin is associated with fragments of pollen plasma membrane remaining on the surface of the sperm cell. This evidence suggests that myosin is not natively present on the surface of the sperm cells, but occurs principally, if not exclusively, on associated membranes that surround the sperm cells; these pollen membranes are largely lost during isolation. The methods used during this study failed to demonstrate significant amounts of functional myosin on isolated sperm cells, suggesting that cells lack sufficient native myosin on their surface to migrate under typical *in vitro* conditions.

Acquisition of functional cytoplasmic myosin is demonstrated by the movement of injected sperm cells and latex beads in *Nitella*. The consistent lack of sperm cell movement: (1) in *ex vitro* assays, (2) in *Nitella* cells lacking intact cytoplasm and (3) with surfaces coated using a neutral "blocking" or positively-charged protein confirms that myosin present on isolated sperm cells is negligible. Isolated sperm cells introduced into actively streaming internodal cells of *Nitella* display actomyosin tracking, therefore demonstrating an ability to acquire superficial myosin even if it is not natively present. Prior research indicates that myosin is present on many, if not all of the membrane-bound organelles in the cytoplasm of *Nitella* (Kachar and Reese 1988). Similar examinations of pollen tube organelles (Miller et al. 1995) and "P-particles" (Heslop-Harrison et al. 1997) provide evidence for myosin associated with

organelles in pollen tubes. Presumably, myosin-associated membranes will undergo normal membrane turnover in the cell, which is one method by which myosin may be exchanged within the cell; however, the possibility that soluble myosin may exist freely within the cytoplasm is supported by dot blots. Solubie myosin that is only transiently associated with the surface of isolated sperm cells and latex beads may be the most significant source of the myosin resulting in the migration of these bodies in *Nitella*; a similar pool of soluble myosin is also evident in cytoplasmic fractions extracted from *Plumbago* pollen.

Experiments altering the surface of sperm cells and latex beads appear to indicate that associated myosin may be stabilized through surface charges. The net charge of sperm cells can be calculated by determining the electrophoretic mobility of the sperm cells using capillary cell microelectrophoresis (Michel et al. 1982) and measuring the viscosity of the solution and the reciprocal of the Debye-Hückel distance (Levine et al. 1983). Exact information on the size, structure and charge of molecules on the cell surface is needed to refine the data further; however, preliminary calculations for *Plumbago* sperm cells indicate that the present results are typical of other measurements of living cells in the literature (Levine et al. 1983; Vargas et al. 1989). To attribute this binding solely to surface charge may be premature, but the current results suggest that electrical charge may be a significant factor in myosin binding on organelle and membrane surfaces. Myosin labeling of pollen tube "P-particles" (Heslop-Harrison et al. 1997) also appears to be related to negative electrical charges

on their surface (Heslop-Harrison and Heslop-Harrison 1982) and therefore this effect may have particular significance in pollen tube function.

That sperm cells of flowering plants appear to be conveyed to their target cells by means of interactions between myosin on the surface of sperm cells and actin bands in the embryo sac (Huang and Russell 1994) gives added significance to the apparent ability of sperm cells to adsorb functional myosin on their surface. Since there is apparently insufficient myosin to support movement of isolated sperm cells prior to their introduction into a myosin-enriched environment, the movement of both sperm cells and latex beads along actin bundles in intact *Nitella* cytoplasm suggests that myosin: (1) can be acquired, (2) is readily adsorbed on their surfaces and (3) may be stabilized by cell surface charge (Michel et al. 1982; Vargas et al. 1989). Two possible sources for such available myosin after the discharge of the sperm cells from the pollen tube include the cytoplasm of the pollen and that of the synergid which receives the sperm cells. Dot blots support the presence of a soluble form of myosin in pollen cytoplasm. Our electrophoretic assays of isolated *Plumbago* sperm cells demonstrated that the surface of the sperm cell is negatively charged, with an average electrophoretic mobility of 1.27 ± 0.23 (μm/sec)/(V/cm). Myosin presumably is adsorbed to the surface of the sperm cells due to opposite charges on the myosin that may stably bind it to the sperm.

An interesting observation is that the electrophoretic mobilities of *Plumbago* sperm cells displayed two different populations (Fig. 7). Human X- and Y-bearing sperm cells also display differing electrophoretic mobilities (Kaneko et al. 1984), reflecting

that different cell functions may play a role in surface characteristics. In *Plumbago*, differences in cell surface charges also appear to reflect sperm cell fates. The S_{sm} -- the sperm cell known to fertilize the egg preferentially (Russell 1994) -- displays greater electrophoretic mobility in sperm pairs. The S_{vn} -- the sperm cell with lesser electrophoretic mobility preferentially fuses with the central cell. Such charge differences could potentially relate to their different fates during fertilization.

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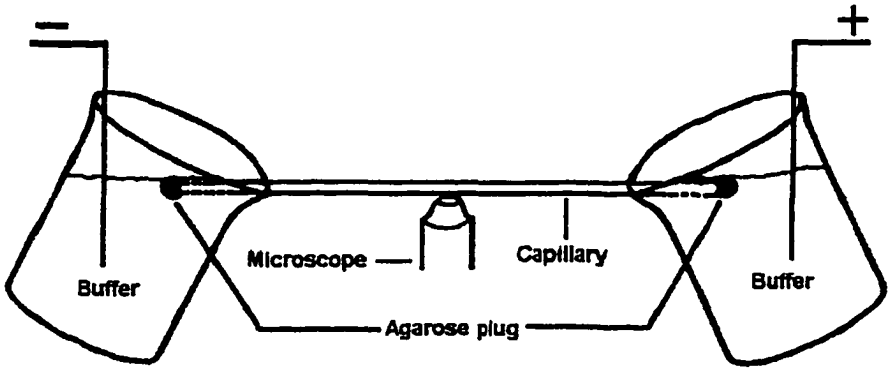

Fig. 1. Diagram of microelectrophoretic apparatus used to calculate the surface charge of the sperm cells of *Plumbago zeylanica.*

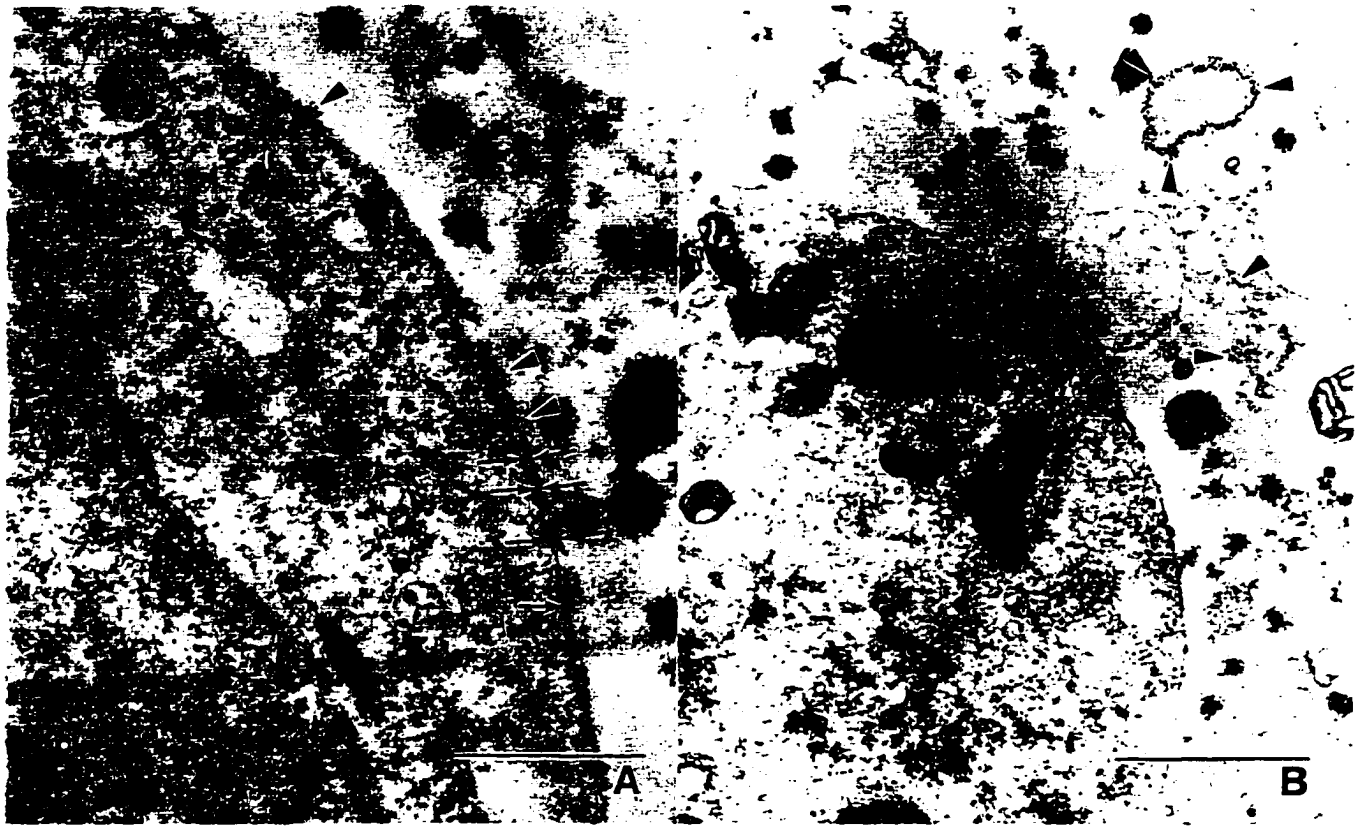

Fig. 2. Immunogold labeling of myosin on isolated sperm cells and organelles of *Plumbago zeylanica*. A. Sparse gold label is associated with adherent membrane fragments (arrowheads) of presumed vegetative cell origin. Arrows pointing toward the right indicate sperm cell plasma membrane; arrows pointing toward the left indicate adherent membrane fragments. Bar = 0.5 μm. B. Sperm cells lacking adherent membrane fragments lack label on the cell surface, although some pollen tube organelles are densely labeled for myosin (arrowheads). Bar = 1 μm.

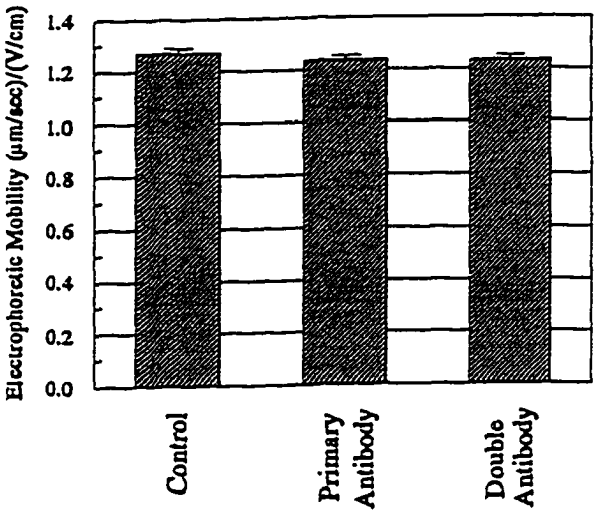

Fig. 3. Electrophoretic mobility of *Plumbago* sperm cells with and without antibody treatment.

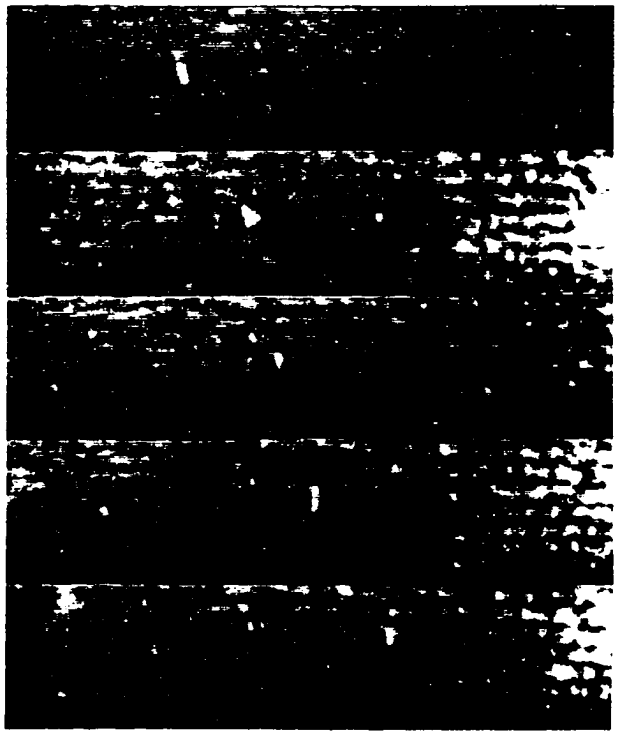

Fig. 4. Time-lapse photomicrographs showing *Plumbago* sperm cell movement in a *Nitella* internodal cell. (Time interval between sequential pictures was 10 s.)

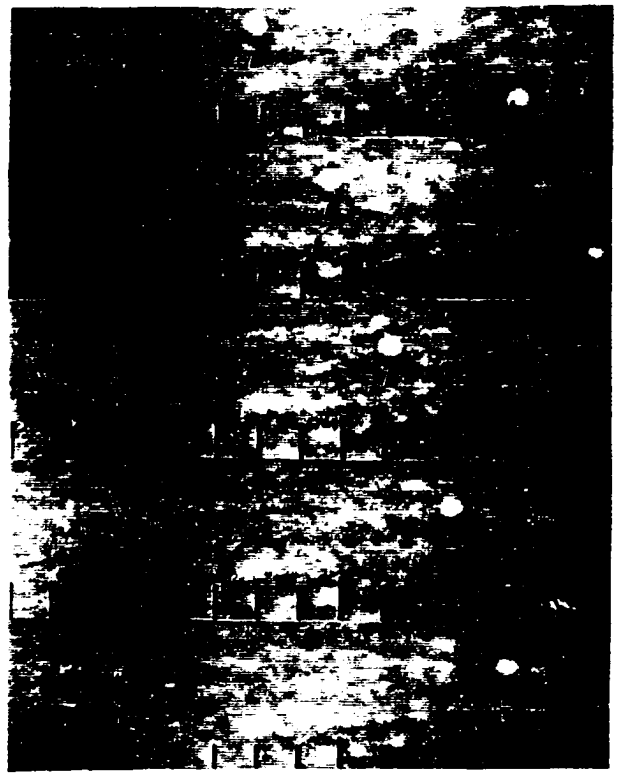

Fig. 5. Time-lapse photomicrographs showing the movement of latex beads in a *Nitella* internodal cell. (Time interval between sequential pictures was 2 s.)

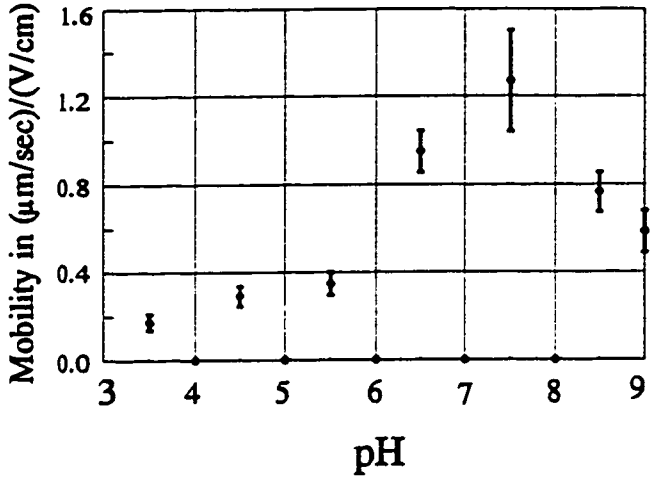

Fig. 6. Electrophoretic mobility of *Plumbago* sperm cells at different pH.

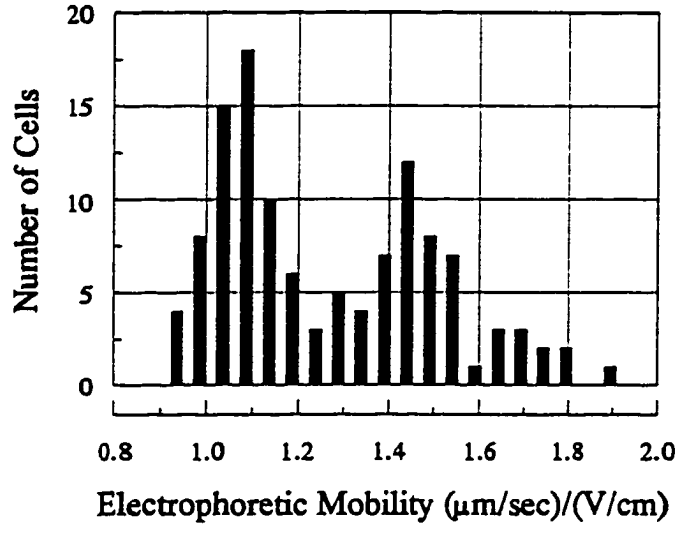

Fig. 7. Electrophoretic mobility of a population of sperm cells of *Plumbago zeylanica.*

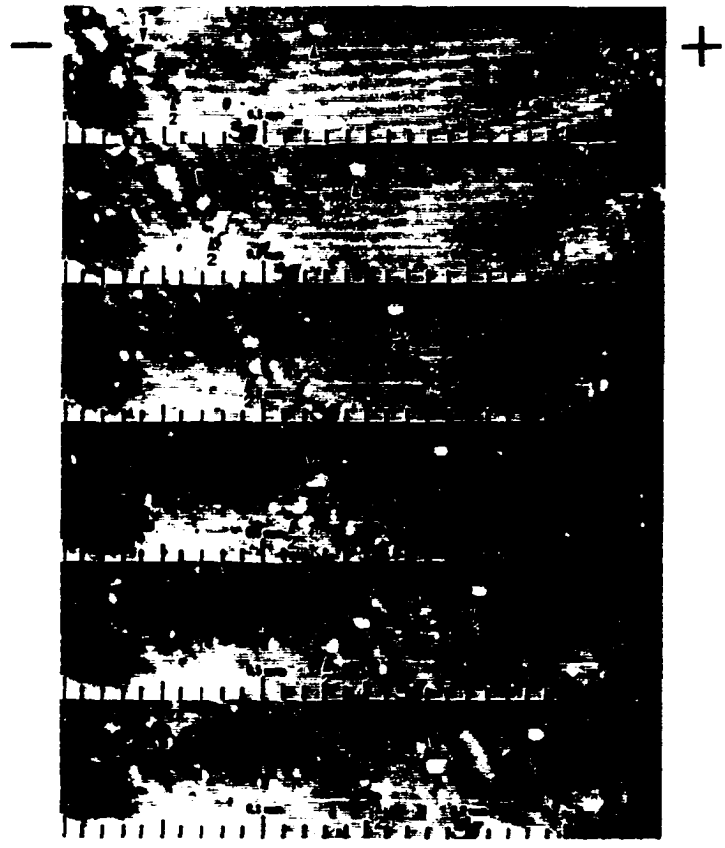

Fig. 8. Movement of *Plumbago* sperm cells in an electric field, showing mobilities of three different sperm cells. (Time interval between sequential pictures was 2 s.)

Fig. 9. Dot blot using anti-myosin antibody showing labeling of soluble components of a highly centrifuged extract of the cytoplasm of (1) *Nitella* internodal cells, (2) *Plumbago* pollen and (3) buffer (control). Control blots omitting primary antibody (not shown) were unlabeled.

CHAPTER 2

Localization of myosin on sperm cell-associated membranes of tobacco (*Nicotiana tabacum* L.)

Summary. Actomyosin interactions are reportedly the principal mechanism for the transport of non-motile sperm cells of flowering plants inside the pollen tube and inside the embryo sac. Myosin has been demonstrated on the generative cell (the predecessor of sperm cells), although it is unclear from previous studies whether myosin is located directly on the plasma membrane of the male germ cells or on the external plasma membrane of the pollen cell that surrounds them. Immunogold scanning electron microscopy was used to localize myosin on isolated tobacco sperm cells, with and without associated membranes. When present, the pollen tube plasma membrane surrounding the sperm cells was labeled by an anti-myosin antibody, as were pollen tube cytoplasmic organelles. Negligible labeling was observed directly on the plasma membrane of the sperm cells.

Keywords: Immunogold; Myosin; *Nicotiana tabacum*; Pollen tube; Scanning electron microscopy; Sperm cell transport.

Introduction

Fertilization in flowering plants involves the transport of sperm cells from the pollen grain, through the pollen tube cell, and ultimately to the female germ cells. Sperm cells are formed either inside the pollen grain (tricellular pollen) or inside the pollen tube (bicellular pollen). Unlike non-seed plants and some gymnosperms, sperm cells in flowering plants are believed to be non-motile, passive participants during their transport in the pollen tube. Sperm cell transport is composed of two phases: (1) transport inside the pollen tube and, (2) positioning of sperm cells inside the embryo sac after the sperm cells are discharged from the pollen tube (Russell 1996).

Evidence suggests that actomyosin interactions may be involved in sperm cell movement inside the pollen tube. Bundles of actin filaments are present in the cortical cytoplasm of the pollen tube (Tang et al. 1989a, Heslop-Harrison and Heslop-Harrison 1989a), and anti-myosin antibody labels the surface of the generative cell—the predecessor of sperm cells (Heslop-Harrison and Heslop-Harrison 1989b, Tang et al. 1989b, Miller et al. 1995, Tirlapur et al. 1995, 1996). Pollen wall-generating vesicles are also labeled by antibodies to myosin (Heslop-Harrison et al. 1997), as are other cytoplasmically transported pollen tube organelles (Miller et al. 1995). The role of actomyosin interactions in male germ cell movement is supported by depolymerization of actin filaments using cytochalasin and inhibition of ATP replenishment (Lancelle and Hepler 1988, Heslop-Harrison and Heslop-Harrison 1989a, 1996). During male germ cell transport inside the pollen tube, the male germ cells are surrounded by their own plasma membrane and, in turn, by the plasma membrane of the pollen tube; the latter membrane separates the sperm cells from

the interior of the tube cell cytoplasm (Yu et al. 1992, Russell 1994, Russell et al. 1996). It was unclear however from prior studies whether myosin was associated directly with male germ cells or with the tube cell plasma membrane.

During migration of the sperm cells following discharge from the pollen tube, the cell in which they are deposited, the synergid, is dramatically altered; two actin bands or "coronas" are formed within the penetrated synergid and between the egg and the central cell (Huang and Russell 1994, Huang and Sheridan 1994). These actin bands appear to trace the pathway taken by the sperm cells during their passage to the egg and central cell. Immunogold labeling of isolated sperm cells of *Plumbago zeylanica*, however, reflects that negligible myosin is present on the plasma membrane of sperm cells (chapter 1).

In the present study, isolated sperm cells of tobacco and immunogold scanning electron microscopy was used to further access the exact location of myosin on sperm cells of angiosperm.

Materials and Methods

Isolation of sperm cells

Plants of *Nicotiana tabacum* L. were grown in a controlled growth chamber at 20-27 °C with 16 hr daylength. Isolation of sperm cells was based on the method of Tian and Russell (1997). Briefly, flowers were hand pollinated 1 d after emasculation. After 30 hr of pollen tube growth, pollinated styles were excised 4 cm from the tip of the stigma. The cut end of the style was immersed in a culture medium containing 0.01% H_3BO_3, 0.01% KH_2PO_4, 0.01% $CaCl_2$ and 15% sucrose (pH 5.4) and grown for about 9 hr at 25

°C. After numerous pollen tubes emerged from the end of the style, the cut end was immersed in a solution containing 9% mannitol (pH 5.4). Most of the pollen tubes burst within 5 min, releasing the sperm cells and the contents of the pollen tubes into the solution.

Scanning electron microscopy

Pollen tubes were burst and sperm cells released from the pollen tubes onto silane coated coverslips (Büechi and Bäechi 1979) placed at the bottom of an Eppendorf microcentrifuge tube. The coverslip was then centrifuged at 1500 × g for 10 min to ensure that the sperm cells were firmly attached. The sample was fixed 40 min with 1% glutaraldehyde in 0.1 M phosphate buffer (pH 6.3), dehydrated in a graded ethanol series, critical-point dried, coated with gold-palladium and examined using an ETEC Autoscan scanning electron microscope at 20 kV.

Immunogold labeling

For immunogold labeling, samples fixed 40 min in 1% glutaraldehyde were blocked using 3% dried milk and 1% fish gelatin in phosphate buffered saline (PBS) (pH 7.3), incubated in a 1:10 dilution of anti-myosin antibody (M-7648, Sigma Chemical Co., St. Louis, MO) with blocking solution for 1 hr, washed three times with blocking solution, and then incubated in a 1:5 dilution of 15 nm gold-conjugated goat anti rabbit IgG (EY Labs, Inc., San Mateo, CA) with blocking solution for 40 min. The specimen was then washed with PBS, dehydrated in a graded ethanol series, critical-point dried, coated with

carbon, and examined using a JEOL JSM-880 scanning electron microscope at 25 kV. Controls were prepared by substituting primary antibody with blocking solution.

Results

Isolated sperm cells appeared ellipsoidal to spherical using scanning electron microscopy (Figs. 1-4). Discharged pollen tube cytoplasm remained largely intact in well-preserved samples (Fig. 1), despite the absence of the outer plasma membrane. The pollen tube plasma membrane around the sperm cells was variably preserved. Fig. 2 illustrates a partially preserved segment of pollen membrane surrounding the sperm cell and an associated cytoplasmic body. The plasma membrane of the tube cell degenerated progressively, first forming a broken sheath, then fine threads or tubules associated with the sperm cells (Fig. 3) and, ultimately disintegrated (Fig. 4). The increasingly rounded shape of the sperm cells was associated with the loss of the surrounding pollen tube plasma membrane. The underlying sperm plasma membrane, however, remained intact (Figs. 2-4).

Filamentous structures associated with the sperm cells (Fig. 5A) displayed dense immunogold myosin labeling, although the underlying sperm cell surface appeared to be labeled only in conjunction with adherent pollen membrane fragments and organelles (Fig. 5B). In sperm cells that lacked surrounding tube cell plasma membranes or had sparse adherent pollen organelles (Fig. 6A), gold labeling was either absent or restricted to these adherent materials (Fig. 6B) and did not occur directly on the sperm cell surface. In Fig. 7A, a sperm cell without adherent pollen organelles appeared to represent a cell

with the surrounding membrane intact (Fig. 7B). Typically, sperm cells that retained their native shapes had intact surrounding membranes, and the whole surface was heavily labeled (Fig. 8). No gold particles were observed in control samples in which the primary antibody was omitted.

Discussion

The movement of angiosperm male germ cells appears to depend on interactions between bundles of F-actin and membrane-associated myosin during their transport in the pollen tube (review: Pierson and Cresti 1992). Among the structures labeling with antibodies to myosin in pollen are the surfaces of generative cells, both in the pollen grain (Heslop-Harrison and Heslop-Harrison 1989b) and, later, in the pollen tube (Tirlapur et al. 1995, 1996). From their inception, the generative cell and later sperm cells are surrounded by pollen plasma membrane, which originates during pollen formation and encloses the generative cell (Russell et al. 1996). The pollen plasma membrane continues to surround the plasma membranes of the generative and sperm cells during later development (Russell 1994); however, in prior studies the precise location of the anti-myosin label could not be distinguished between the pollen and male germ unit membranes. The earliest indication in the published literature that myosin was located on the ensheathing pollen plasma membrane was reported by Heslop-Harrison and Heslop-Harrison (1989b), who noted that only some of the generative cells examined were labeled with anti-myosin antibodies. They noted that labeled cells appeared to be associated with an external membrane and postulated that generative cells

that were unlabeled may have lacked this membrane (Heslop-Harrison and Heslop-Harrison 1989b).

The current study appears to confirm this finding, since anti-myosin labeling is localized on pollen cell plasma membranes. Those sperm cells lacking adherent pollen membranes are essentially unlabeled. Immunogold transmission electron microscopic observations of *Plumbago* sperm cells have revealed a similar localization of anti-myosin label on the pollen plasma membrane but not on the sperm cell surface (chapter 1).

The loss of the surrounding pollen tube plasma membrane is an expected consequence of pollen tube discharge during normal fertilization (Russell 1994), which also occurs during sperm cell isolation (Russell 1991). Although actomyosin migration of sperm cells to the female target nuclei is still possible, the sperm cells would have to obtain myosin from other sources after their release into the embryo sac (Zhang and Russell 1995, Russell 1996).

Acknowledgments

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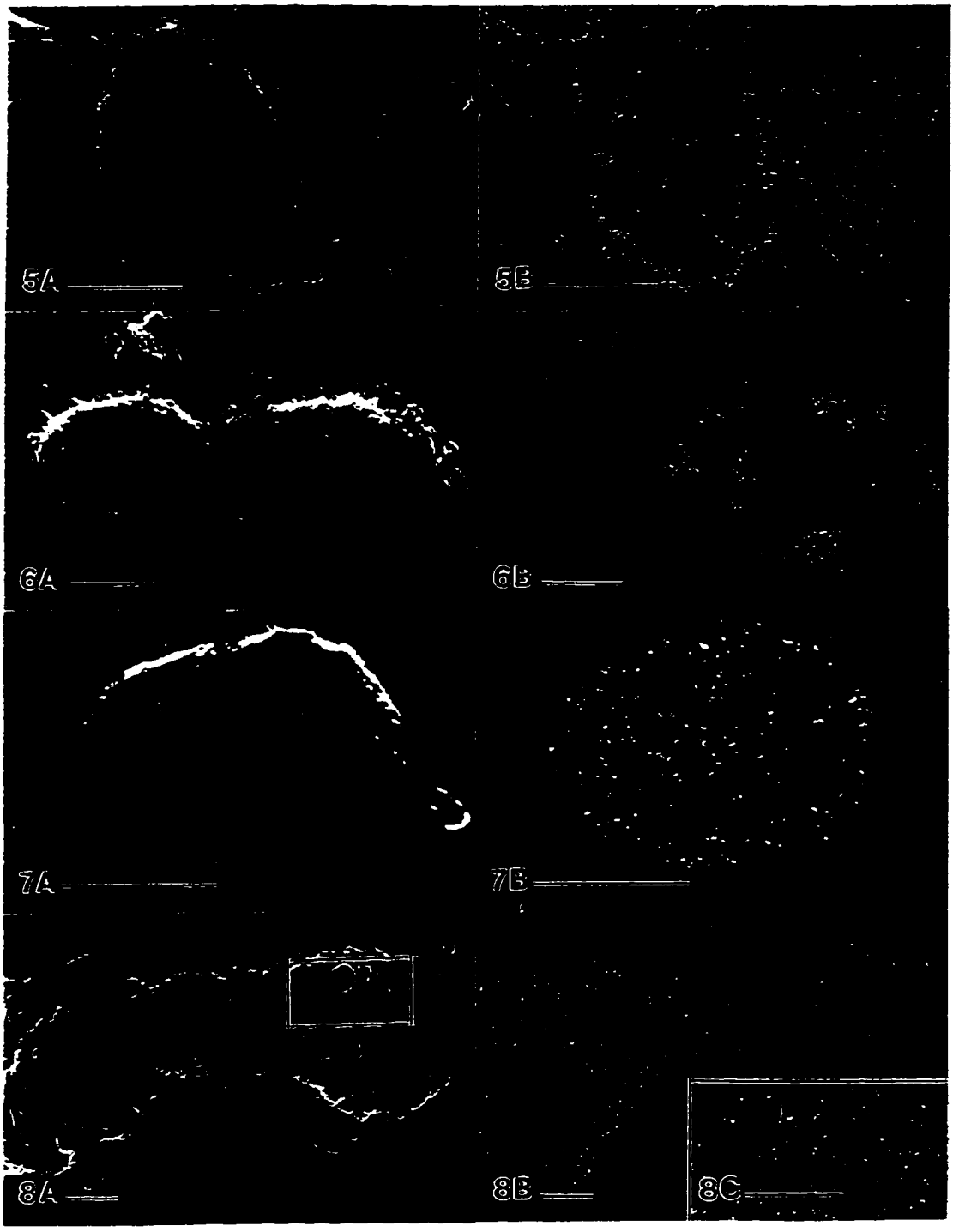
Figure 1. Scanning electron micrograph of an isolated sperm cell and associated pollen tube cytoplasm. Bar: 5 μm.

Figures 2-4. Scanning electron micrographs of isolated sperm cells, showing an enveloping membrane (arrowheads) around the sperm cells (Fig. 2). The membrane later may become filament-like (arrowheads) after isolation (Fig. 3), with the membrane eventually disappearing (Fig. 4). Bars: 2 μm.

Figures 5-8. Immunogold labeling of myosin on isolated sperm cells and organelles. 5A. Secondary electron image (SEI), showing a sperm cell and surrounding filamentous structures. 5B. Backscattered electron image (BEI) of the same field as in Fig. 5A, showing filament-like structures and organelles on the sperm cell labeled with gold particles, but no label on the sperm plasma membrane. 6A. SEI of a sperm cell pair isolated from the same pollen tube. The left sperm cell lacks surrounding pollen membrane. 6B. BEI of the same field as in Fig. 6A, showing absence of labeling on the plasma membrane of sperm cells, whereas some organelles and adherent membrane fragments on the sperm cells are heavily labeled. 7A. SEI of an isolated sperm cell with associated pollen membranes. 7B. BEI of the same field as in Fig. 7A shows surrounding membrane labels for myosin. 8A. SEI of two sperm cells surrounded by a pollen plasma membrane. 8B. BEI of the same field as in Fig. 8A, showing the membrane labeled with gold particles. 8C. BEI of the same field as inside the box of Fig. 8A, showing labeled gold particles at larger magnification. Bars: 1 μm.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CHAPTER 3

Microelectrophoresis of pairs of sperm cells isolated from single pollen tubes of *Nicotiana tabacum*

Abstract Microelectrophoretic analysis of paired sperm cells isolated from single pollen tubes of *Nicotiana tabacum* was conducted at four different developmental stages. The two sperm cells associated in a male germ unit are negatively charged at all developmental stages examined, but when separated, only newly formed sperm cells (stage 1) were negatively charged. Isolated sperm cells at stages 2, 3, and 4 were positively charged under current assay condition. The S_{us} has a greater electrophoretic mobility than the S_{vn} in all developmental stages. Sperm cells isolated from stage 1 are most sensitive to electrical damage. Sperm cell surface charge differs between the S_{us} and S_{vn}, suggesting that the two sperm cells may display preferential fusion during fertilization.

Key words Microelectrophoresis · *Nicotiana tabacum* · Pollen tube · Preferential fertilization · Sperm cells · Surface charge

Introduction

In angiosperms, two sperm cells are involved in double fertilization; one fuses with the egg cell to form the embryo and the other fuses with the central cell to form the nutritive endosperm. The two sperm cells are typically connected to one another by an inner pollen membrane and physically associated with the vegetative nucleus, forming the functional transmission unit of sexual reproduction in flowering plants known as the male germ unit (MGU) (Dumas et al. 1984). The linkage of the sperm cells increases the effectiveness of gamete delivery in the pollen tube, and ensures nearly simultaneous transmission of the two sperm cells into the receptive embryo sac (Russell and Cass 1981). After entering the embryo sac, the two sperm cells separate from their association and fuse with their individual target cells.

Differences of the two sperm cells have been reported in a number of angiosperms (Russell 1991) and these differences may potentially relate to preferential fertilization in which one sperm cell has a greater likelihood of fusing with the egg (Russell 1992). In *Plumbago zeylanica*, for example, only the sperm cell unassociated with the vegetative nucleus (S_{ua}) contains plastids and the sperm cell physically associated with the vegetative nucleus (S_{va}) contains nearly five times as many mitochondria (Russell and Cass 1981; Russell 1984). Fusion between the egg and the plastid-rich sperm cell (S_{ua}) occurs in 94% of the cases examined (Russell 1985). In plants in which there is not a clear marker for fusion, however, the polarity of fertilization is difficult to determine. Electrophoretic analysis of sperm cells of *Plumbago zeylanica* showed that the two

sperm cells have different surface charge densities (Chapter 1), indicating that differences in surface charge is correlated with preferential fertilization.

Since the work of Shivanna et al. (1988), there has been some doubt about whether sperm cells are completely mature before germination of the pollen grain and whether they possess any evident physiological limitations. Faure et al. (1994) demonstrated that isolated sperm cells can be induced to fuse with isolated egg cell and the products of fusion can be used to regenerate fertile plants (Kranz and Lörz 1993), suggesting that they are mature. The sperm cells used in these experiments were isolated from newly released pollen grains, indicating that sperm cells are fully functional at anthesis in these plants. In bicellular pollen-producing plants, however, the sperm cells are formed in the pollen tube, and therefore the timing of maturity and acquisition of physiological competence of sperm cells is still unclear (reviews: Russell 1991; Theunis et al. 1991).

In this study, microelectrophoresis of paired sperm cells isolated from single pollen tubes of tobacco (*Nicotiana tabacum*) at different developmental stages was performed in order to further understand the surface charge of sperm cells and its relationship to preferential fertilization in flowering plants.

Materials and methods

Isolation of sperm cells: Plants of *Nicotiana tabacum* L. were grown in a controlled growth chamber at 20-27°C with 16 h daylength. Flowers were emasculated one half day before anthesis and sperm cell isolated using the method of Tian and Russell (1997).

Briefly, flowers were hand pollinated 1 d after emasculation. After 12-32 h growth, the styles were excised 1, 2, 3, or 4 cm from the tip of the stigma (Tian and Russell 1997). The cut ends of styles were then immersed in a culture medium containing 0.01% H_3BO_3, 0.01% KH_2PO_4, 0.01% $CaCl_2$ and 15% sucrose, pH 5.4, and grown for 6-9 h at 25°C. After numerous pollen tubes emerged from the end of the style, the style was immersed in a solution of 9% mannitol. Most of pollen tubes burst within 5 min and pairs of sperm cells were released from pollen tubes into solution.

Cell microelectrophoresis: A microelectrophoretic chamber was constructed using glass and super glue to build a 5 mm wide, 6 cm long trough. Both ends of the trough were sealed with 2% agarose forming a long narrow chamber. The chamber was then glued into a Petri dish. 2% agarose was applied inside the Petri dish along the two ends of the chamber, forming two reservoirs. The chamber and the reservoirs were then filled with low ionic buffer containing 10 mM Na_2HPO_4, 0.3 mM citric acid and 9% mannitol (pH 6.0) with an electrode immersed into each reservoir. A pair of sperm cells isolated from the same pollen tube was transferred into the buffer in the center of the optical field within the chamber by a microinjector. Selected voltages were applied between the electrodes with constant current; the mobility of the sperm cells was observed using an inverted phase contrast microscope. Images were recorded using a high sensitivity television camera and a video recorder. The velocity was measured using the recorded images. The electrophoretic mobility and surface charge of sperm cells were calculated according to methods described in Chapter 1.

Results

Newly released sperm cells from single pollen tubes appeared to remain associated for the first 5 min, after which the two cells start to separate. The vegetative nucleus, which is about 15 μm in diameter, is usually associated with one of the sperm cells when the sperm cells are released. The vegetative nucleus is unstable under isolation conditions, possibly in part due to osmotic pressure. Newly-released sperm cells are ellipsoidal and elongated. As they separate, the cells became football shaped and then round. The two sperm cells were different in size: the average diameter of the mature S_{vn} was 6.73 μm, whereas the mature S_{us} was 7.52 μm (Fig. 1).

When associated in a MGU, isolated sperm cells always migrated toward the positive pole in the electrophoretic chamber, regardless of developmental stage, indicating that the MGU surface was net negatively charged. The electrophoretic mobility of the MGU was highest at stage 3, with a value of -1.77 ± 0.20 (μm/sec)/(V/cm) (Table 1).

Isolated, separated sperm cells, however, migrated toward the positive pole only at stage 1. Sperm cells isolated from stages 2, 3, or 4 migrated toward the negative pole of the electrophoretic chamber, indicating that they carried a net positive charge on their surface. In all developmental stages, regardless their direction of migration, the two sperm cells differed in their electrophoretic mobilities, with the larger S_{us} always having a higher electrophoretic mobility than the S_{vn}. Figure 2 illustrates a pair of isolated sperm cells from stage 4 moving toward the negative pole of the electrophoretic chamber at different velocities. Stage 1 sperm cells are particularly sensitive to the electrical

potential, as many of them burst after 5 min of applying the potential. Organelles released from pollen tubes are negatively charged and move faster than the sperm cells.

The calculated surface charges of sperm cells at different developmental stages are shown in Figure 3.

Discussion

Preferential fertilization has been proven to occur in only a few species of flowering plants in which the sperm cells show traceable differences (Russell 1991). These differences mainly include: (1) differences in cytoplasmic organelles and size (cytoplasmic heterospermy) and (2) differences in nuclear complement in the sperm cells (nuclear heterospermy) (Russell 1985). The most common form of cytoplasmic heterospermy consists of iniquities in mitochondria between the two sperm cells, with the S_{vn} usually containing more mitochondria (Russell 1992). Nuclear heterospermy has been reported only in *Zea mays*, where B chromosomes often do not segregate during generative cell division, resulting in aneuploid sperm cells (Roman 1948). In *Plumbago zeylanica*, both mitochondria and plastids differ between the two sperm cells. The S_{vn} usually contains no plastids and more than 200 mitochondria, whereas the S_{us} contains an average of 24 plastids and less than 50 mitochondria. During fertilization usually the S_{us} fuses with the egg cell and the S_{vn} fuses with central cell (Russell 1984). Microelectrophoresis of *Plumbago* sperm cells showed that S_{vn} and S_{us} carry different surface charges (chapter 1). In the current study, tobacco sperm cells also show difference in cell surface charge (surface heterospermy). Such surface heterospermy

suggests the possibility of preferential fertilization exists in *Nicotiana*. The S_{us} of both *Plumbago* and *Nicotiana* has a higher mobility than the S_{vn}. Whether this indicates that the same two sperm cells in different species may have similar fates during fertilization, however, remains to be determined (Table 2). Preliminary *in vitro* fusions using isolated egg and sperm cells of tobacco demonstrated a higher tendency for the S_{us} and egg cell to fuse (Tian and Russell unpublished data) which support this contention.

Two types of association are evident in the MGU. One is the association between the vegetative nucleus and the generative cell and sperm cells, whereas the other is the association between the two sperm cells, which is initiated at generative cell division. The association of sperm cells is based on (1) the initial sharing of the sperm cell common wall (formed during generative cell division); and (2) the inclusion of the two sperm cells within a shared inner plasma membrane which delineates the surrounding vegetative cell. The existence of this inner plasma membrane is reflected by the significant surface charge difference between MGU and single sperm cells.

As a functional unit, the MGU facilitates the transmission of sperm cells in pollen tube (Dumas et al. 1984). Upon arrival in the embryo sac, the pollen tube discharges its contents into the receptive synergid. Sperm cells are deposited into the embryo sac, losing their external surrounding membrane (Huang et al. 1993). With the disruption of the MGU, the sperm cells, therefore, are transmitted individually from the receptive synergid to their target cells. This transmission mechanism may differ with that in pollen tube, as supported by the surface charge differences between the MGU and the separated sperm cells.

In tricellular pollen, Zhang et al. (1993) and Matthys-Rochon et al. (1994) showed that sperm nuclei are transcriptionally active, display electrophysiological activity and appear to be fully physiologically competent. *In vitro* fusion of isolated sperm cells and egg cells (Faure et al. 1994) and the regeneration of *in vitro* fusion products (Kranz and Lörz 1993) further indicate that sperm cells newly released from pollen grains are fully functional. In bicellular pollen, however, fewer studies have been reported. In *Nicotiana*, a quantitative cytological study of the sperm cells (Yu and Russell 1994) suggested that most of the sperm cell parameters remained unchanged during the pollen tube elongation, with the exception of a significant increase in the volume of the sperm cells. The fusion frequency of *Nicotiana* sperm cells at different developmental stage decreased with continued pollen tube elongation (Tian and Russell 1998). Microelectrophoresis of tobacco sperm cells also showed a significant difference between the newly formed sperm cells (stage 1) and the mature ones (stage 4). The newly formed sperm cells are more sensitive to electrical potential and showed a tendency for spontaneous self-fusion (Tian and Russell 1998), also suggesting the immaturity of their membranes. The sperm cells probably reach maturity in terms of surface charge at stage 3, as indicated in Figure 3, in which sperm cell surface increased in negative charge from stage 1 to stage 3.

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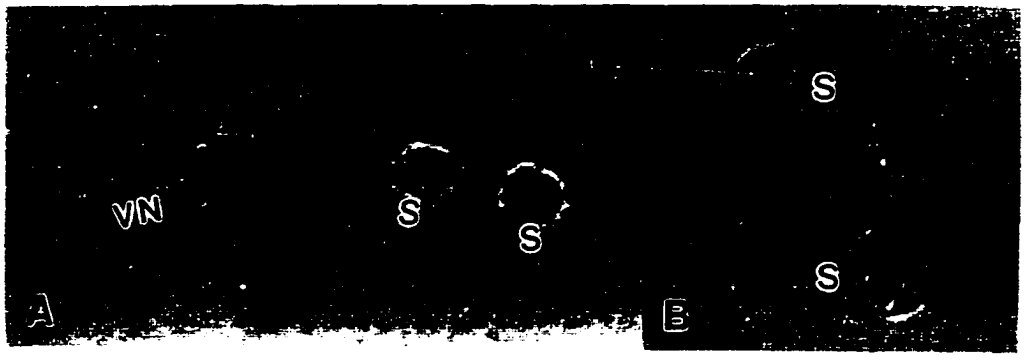

Fig. 1. A pair of sperm cells isolated from stage 4.

A. Two sperm cells (S) and the vegetative nucleus (VN), showing the linkage between sperm cells and vegetative nucleus.

B. The separated sperm cells of the MGU, differ in size.

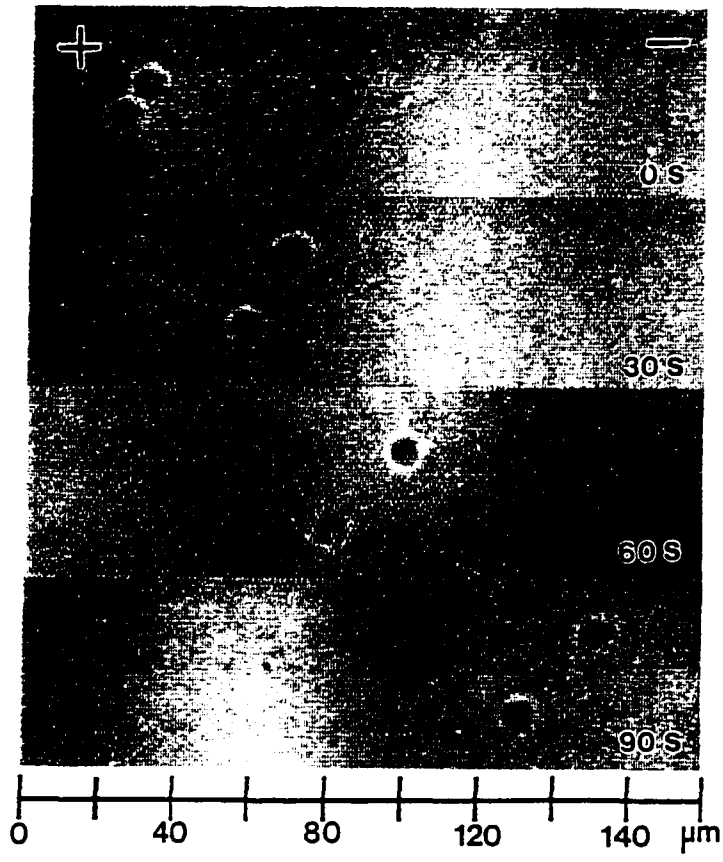

Fig. 2. Microelectrophoresis of separated sperm cells from a single pollen tube demonstrates that sperm cells migrate toward the negative pole at different velocities

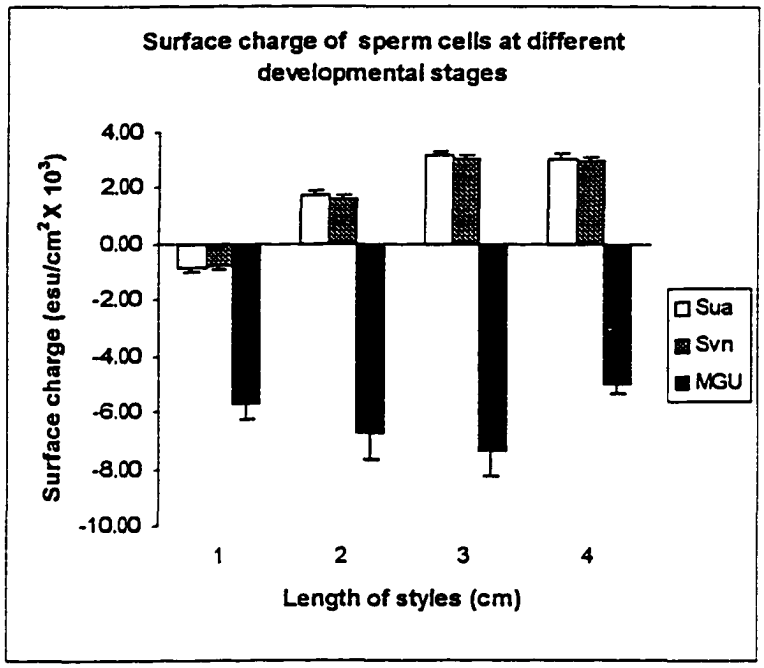

Fig. 3. Comparison of sperm cell surface charge at different developmental stages. N=15 for each dataset.

Table 1: Electrophoretic mobility ((μm/sec)/(V/cm)) of sperm cells of *Nicotiana tabacum* at four developmental stages.

	Electrophoretic mobility ((μm/sec)/(V/cm))			
	Stage 1	***Stage 2***	***Stage 3***	***Stage 4***
MGU	-1.37 ± 0.14	-1.63 ± 0.21	-1.77 ± 0.20	-1.15 ± 0.10
S_{vn}	-0.19 ± 0.03	0.39 ± 0.04	0.72 ± 0.04	0.71 ± 0.04
S_{un}	-0.21 ± 0.02	0.43 ± 0.03	0.75 ± 0.04	0.73 ± 0.04

Table 2. Comparison of characteristics of *Plumbago* isolated for pollen grains at anthesis and *Nicotiana* sperm cells collected at stage 4, which is believed to represent a mature stage

Plant	Sperm type	Size (μm)	Surface Charge (esu/cm² × 10³)	mobility (μm/sec)/(V/cm)	Fate in fertilization
Plumbago	S_{us}	6.91	− 8.28	1.42	Fuse with egg cell
	S_{vn}	7.98	− 6.12	1.05	Fuse with central cell
Nicotiana	S_{us}	7.52	+ 3.03	0.73	Unknown
	S_{vn}	6.73	+ 2.94	0.71	Unknown

CHAPTER 4

Isolation and collection of two populations of viable sperm cells from the pollen of *Plumbago zeylanica*

Summary

A protocol is described for individually collecting two populations of sperm cells, S_{vn} and S_{ua}, from pollen of *Plumbago zeylanica*. Pollen grains were burst in 10 mM MOPS buffer containing 9% mannitol (pH 4.6). Paired sperm cells released from pollen were separated using a microinjector. S_{vn} and S_{ua} were then collected individually with a microinjector, based upon known size differences. Collected sperm cells were washed with isolation medium and transferred to liquid nitrogen until use. FCR test of isolated sperm cells showed a positive reaction, indicating that the isolated sperm cells are viable; most of the sperm cells retain viability for at least 2 h.

Key words: *Plumbago zeylanica*, pollen, preferential fertilization, sperm cell isolation

Introduction

Fertilization in flowering plants involves two sperm cells -- one sperm cell fuses with the egg cell to form the embryo and the other fuses with the central cell to form the endosperm, a nutritive tissue needed for growth of the succeeding generation. Differences in the two sperm cells have been reported in a number of angiosperms (Russell, 1991). In a number of plants, sperm dimorphism has been found that may relate to preferential fertilization, in which one sperm cell has a greater likelihood of fusing with the egg (Russell, 1992). In *Plumbago zeylanica*, the two sperm cells differ in their content of both mitochondria and plastids. The sperm cell physically associated with the vegetative nucleus (S_{vn}) frequently contains no plastids and more than 200 mitochondria, whereas the sperm cell unassociated with vegetative nucleus (S_{ua}) contains an average of 24 plastids and less than 50 mitochondria (Russell, 1984). Fusion between the egg and the plastid-rich sperm cell (S_{ua}) occurs in 94% of the cases examined (Russell, 1985). The molecular mechanism that causes the two sperm cells to differentiate as well as participate in preferential fertilization is unknown. In order to study potential gene involvement in preferential fertilization, highly purified viable sperm cells of both the S_{vn} and S_{ua}, as separate populations, are needed.

In contrast with readily accessible animal sperm cells, the unique "cell-within-a-cell" construction of plant sperm cells poses difficult problems for their isolation and further manipulation. In addition, the two sperm cells from a single pollen grain are, in some circumstances, difficult to discriminate and collect separately from one another. Preliminary work has demonstrated that pollen cytoplasmic organelles may represent a major contaminant for mRNA/DNA analysis of sperm cells (Xu, Russell, Zhang and

Singh, unpublished data). In this paper, we report a protocol to individually collect two populations of viable sperm cells from pollen of *Plumbago zeylanica* (S_{ua} and S_{vn}), which are especially adapted for molecular study.

Materials and methods

Plants of *Plumbago zeylanica* L. were grown in a greenhouse at the University of Oklahoma. Fresh pollen collected from flowers was immersed into a solution containing 10 mM MOPS and 0.8 M mannitol (pH 4.6) to release the sperm cells. Most of the pollen bursts due to osmotic shock within 5 min, releasing the osmotically more tolerant sperm cells, vegetative nuclei and pollen contents into the solution. Paired sperm cells released from the same pollen grain are separated using a microinjector (Effendorf, type 5242) using flame-drawn capillaries, trimmed to an aperture size of 15 μm. S_{ua} and S_{vn} were then collected in individual groups using the microinjector, based upon the their size difference, which known to be consistent based on past study (Russell 1984), omitting pairs without a conspicuous size difference. Unpaired cells were omitted because the sperm size ranges overlap over part of their range. Collected sperm cells (S_{vn} or S_{ua}) were then washed two times with the isolation medium to eliminate pollen cytoplasmic organelles. The purified sperm cells were then transferred into an Eppendorf microcentrifuge tube, which was pre-cooled with liquid nitrogen. The sperm cells were stored in liquid nitrogen or dry ice until use.

The viability of collected sperm cells was evaluated by fluorochromatic reaction (FCR) (Heslop-Harrison and Heslop-Harrison, 1970), using 0.0002% fluorescein diacetate (FDA). The FDA was mixed as 0.1% (w/v) FDA in acetone to form a

solution, which was then diluted 1:50 with water; 10 µl of the diluted stock was used for each 100 µl of isolation solution).

Results

The newly released sperm cells from pollen are generally paired and normally spindle shaped (Fig. 1*a*). The sperm cells rapidly become ellipsoidal to nearly spherical, but are still connected by membranous surrounding materials, often including the inner pollen plasma membrane. The distance between the two sperm cells increases with time after isolation. The average diameters of the S_{vn} and S_{sa} are 7.98 ± 0.50 µm and 6.91 ± 0.44 µm, respectively (Fig. 1*b*). The vegetative nucleus is frequently observed within a few minutes of isolation associated with the sperm cells, forming the so-called male germ unit (Dumas *et al.*, 1984) (Fig. 1*a*). Vegetative nuclei were excluded from harvest, however, because of their fragility and large size, which matched that of the microinjector pipette tip (15 µm diameter).

Newly-released sperm cells initially aggregate with pollen cytoplasm forming clusters that are difficult to separate with a microinjector (Fig. 2*a-c*). As the sperm cells are transferred from the isolation medium to the first wash, sperm cells readily separate from much of the surrounding pollen material (Fig. 2*d*), although some cells still have pollen organelles associated with them. With the second wash, most of the organelles have been eliminated (Fig 2e). As the sperm cells are purified further, they tend to aggregate in solution, separated by a 200--500 nm perimeter (Fig. 2*e*). At this stage, the contamination is reduced to less than 1 organelle/sperm cell. Collection efficiency can routinely reach nearly 100 sperm cells per hour with excellent purity. The sperm cells

display a positive FCR indicating that isolated sperm cells are viable (Fig. 3). Sperm cells retain viability for at least 2 h.

Discussion

The first attempt to isolate sperm cells from angiosperms was reported by Russell (1986), who used 20% sucrose as bursting solution and obtained an enriched fraction of sperm cells from the pollen of *Plumbago zeylanica*. Viability, as measured using the FCR test was less than 5 min in this solution, although Evan's blue continued to be excluded from sperm cells for up to 24 h; this suggests that cell membranes are largely intact, but that cell integrity is in some ways compromised. The protocol reported here results in FCR positivity for more than 2 h after isolation of sperm cells. This is indicative of a significant improvement in the quality of the sperm cells of *Plumbago*, since FCR-positivity appears to be required for *in vitro* fertilization (Kranz *et al.*, 1993; Faure *et al.*, 1994) and is highly desirable for other methods of experimental manipulation.

Numerous protocols have emerged for isolation of plant sperm cells en masse (Shivanna *et al.*, 1988; Southworth and Knox, 1988; Russell, 1991; Theunis *et al.*, 1991). En masse isolation of sperm cells involves either centrifugation on a discontinuous gradient or filtration using a polycarbonate filter. In none of these protocols, however, are sperm cells so highly purified from contaminating pollen organelles nor could individual types of sperm cells (S_{vn} and S_{ua}) be collected into separate populations. Using the highly purified populations of sperm cells from each morphotype (S_{vn} and S_{ua}), as described here, will improve the utility of such sensitive

techniques, as immunoblotting, elicitation of monoclonal antibodies or the preparation of PCR-amplified cDNA libraries. The use of these techniques in sperm cell biology will enhance our understanding of the unique properties that the male gametes of angiosperms may possess and contribute to an understanding of the nature of preferential fertilization.

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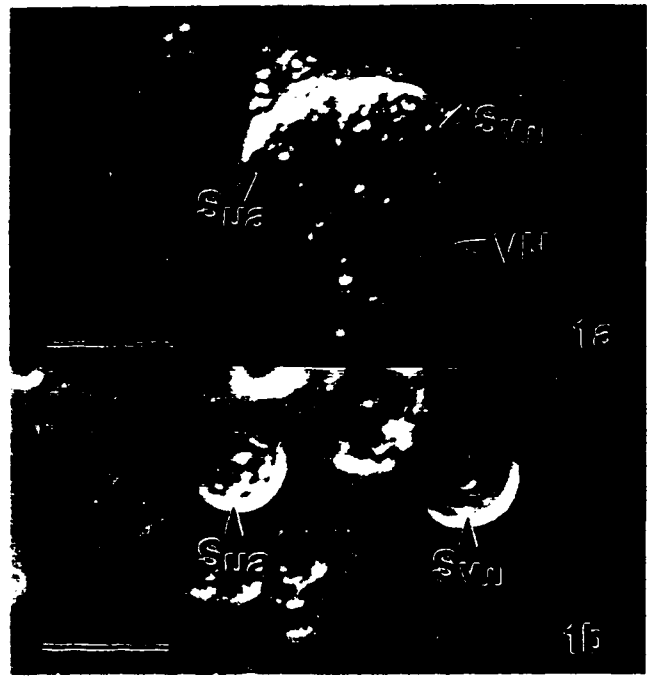


Figure 1 Interference contrast micrograph of a pair of sperm cells isolated from pollen of *Plumbago zeylanica*. (*a*) Newly-released sperm cells (S_{ua} and S_{vn}) associated with the vegetative nucleus (VN). (*b*) Five minutes later, the sperm cells have separated from each other and become spherical. The S_{vn} is the larger of the two cells and is initially physically associated with the vegetative nucleus. Scale bars represent 10 μm.

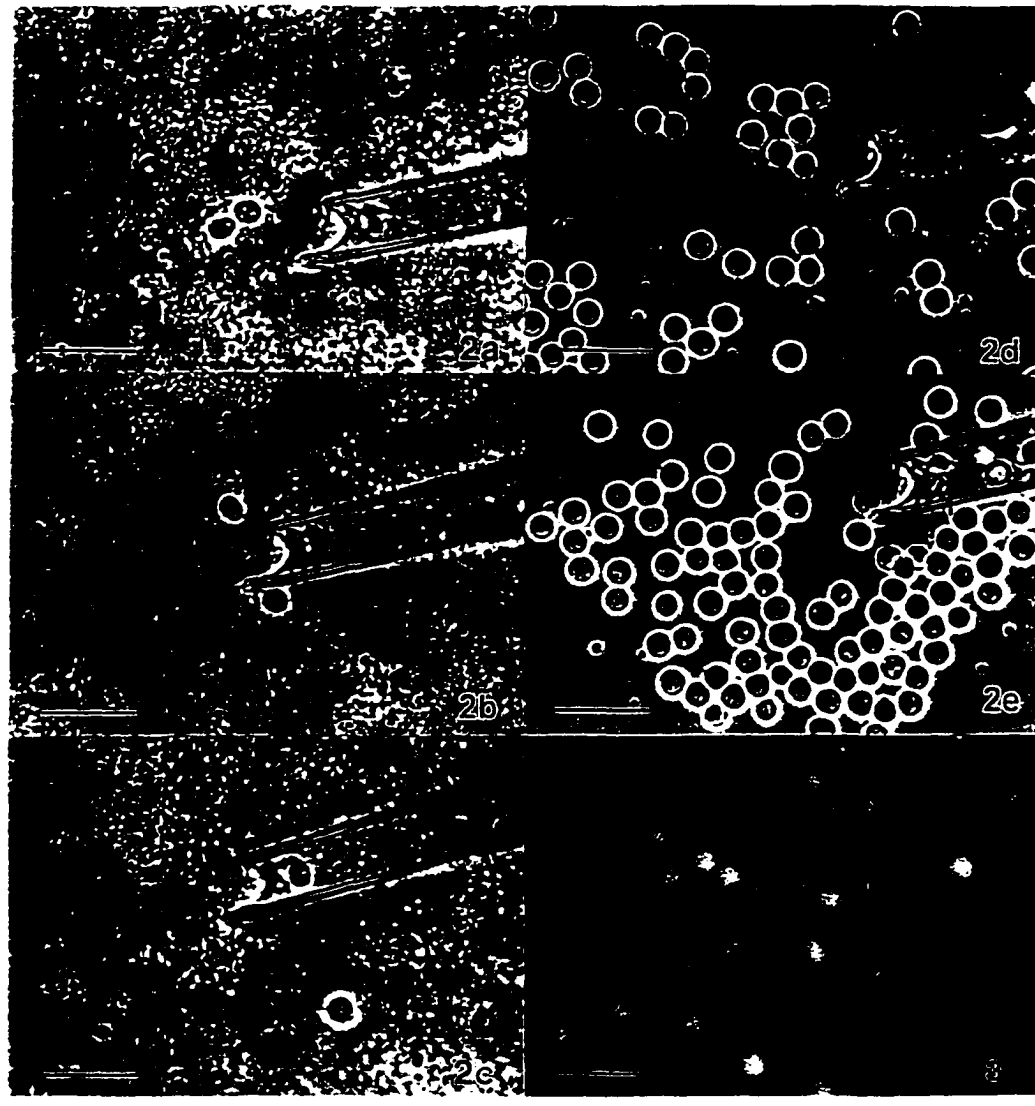
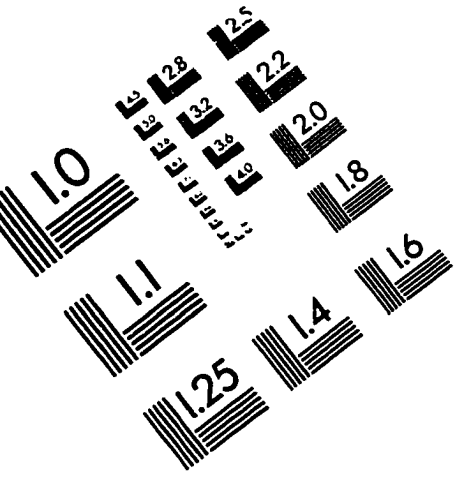
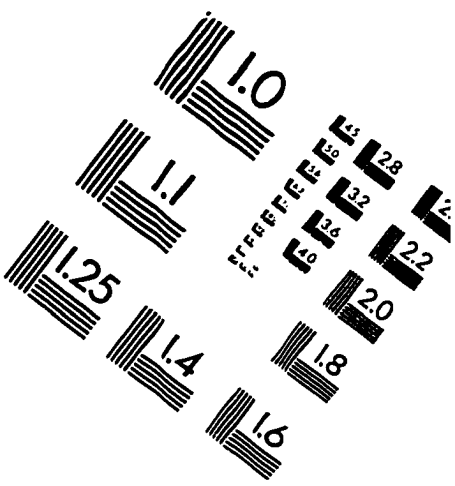


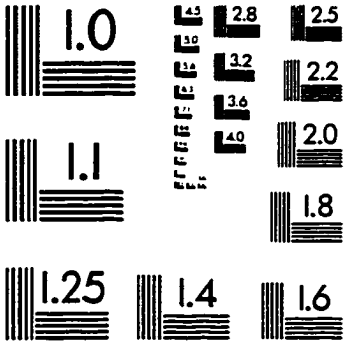
Figure 2 Collection of two populations of sperm cells with a microinjector. (*a*) Two sperm cells released from the same pollen grain are found in pairs. (*b*) Sperm cells are separated with the microinjector. (*c*) One sperm cell (S_{ua}) is drawn into the microinjector. (*d*) First wash of collected sperm cells (S_{ua}). (*e*) Second wash of collected sperm cells (S_{ua}). Organelles left in solution are less than one organelle/sperm cell. Scale bars represent 30 μm.

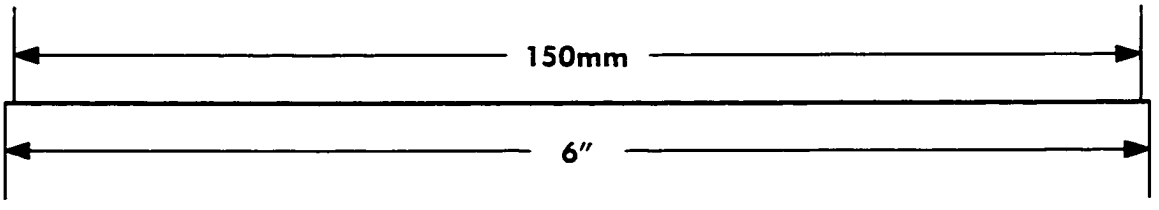
Figure 3 FCR test of isolated sperm cells (S_{ua}), showing a positive reaction with epifluorescence microscopy. Scale bar represents 30 μm.

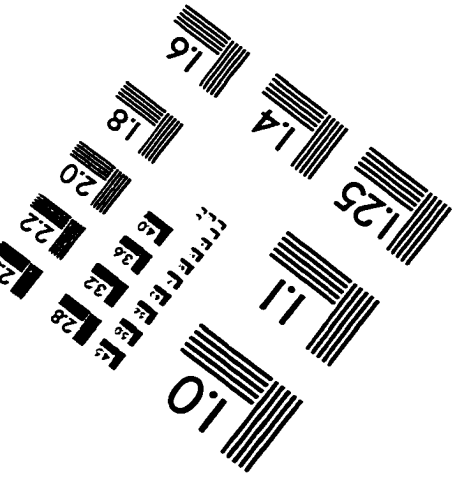
IMAGE EVALUATION
TEST TARGET (QA-3)

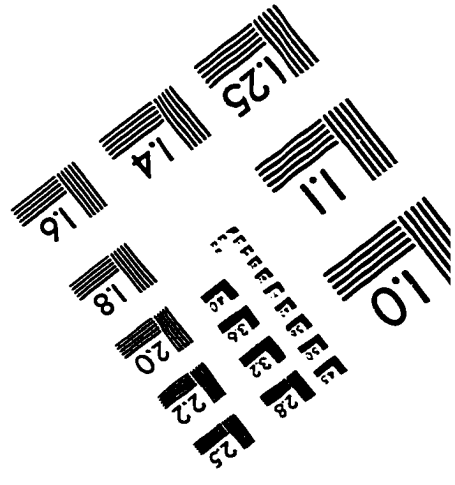












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